The Relevance of CD44 and Hyaluronan Interaction in Osteosarcoma Progression and Metastasis

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Ana Gvozdenovic

aus

Serbien

Promotionskomitee

Prof. Dr. François Verrey (Vorsitz)

Prof. Dr. Bruno Fuchs

Prof. Dr. Ivan Stamenkovic

PD Dr. Lubor Borsig

Zürich, 2012

Contents

1 Summary

Formation of metastases in the lungs is the major cause of death in patients suffering from osteosarcoma (OS), the most common primary bone cancer in children and adolescents. Significant clinical improvements over the past several decades through the use of combination chemotherapy and surgery have led to a dramatic increase in the survival of patients with localized disease. However, patients with metastatic or recurrent disease continue to have a very poor prognosis, with <20% long term survival. The failure of treatment in these patients is often associated with gained resistance of tumor cells to chemotherapy. Therefore, it is of substantial relevance to identify molecular markers associated with the increased metastatic potential or chemoresistance, which may serve as diagnostic or prognostic factors. Acquiring insight into the basic biology of OS progression will make the identification of such new therapeutic targets possible with the final goal to develop treatment strategies that eradicate metastases and thereby improve the survival of OS patients.

Metastasis is a complex multistep process which involves detachment of tumor cells from primary tumor mass, invasion of local stroma, intravasation, survival during transport in the circulation, arrest at distant organ sites, extravasation into corresponding parenchyma, adaptation to this new foreign tissue microenvironment and finally colonization in the distant organ. CD44 has been linked with increased metastatic spread in various types of cancer, however, its function in sarcomas has been only superficially addressed. CD44 and its numerous splice variants constitute a family of widely distributed type I transmembrane glycoproteins that serve as cell-cell and cell-matrix adhesion molecules and as principal

receptors for hyaluronan (HA), a major component of the extracellular matrix in many tissues including bone.

In this thesis, the prognostic value of CD44 expression for OS patients' outcome and the biological relevance of CD44/HA interactions for *in vitro* malignant properties of OS tumor cells and for *in vivo* OS progression and metastasis in orthotopic xenograft OS mouse models were investigated.

Our tissue microarray analysis of OS tumor specimens identified CD44 expression as an additional indicator of poor prognosis together with metastasis and resistance to chemotherapy, the two best established predictors of poor OS patient's outcome.

In vitro, CD44 expression correlated with the adhesion to HA and with cell migration. Moreover, cells with forced CD44 expression were more resistant to cisplatin.

In vivo, our studies in different human xenograft OS mouse models revealed that CD44 gene products may play a dual role in regulating OS progression and metastases, depending on the cellular background. However, in the context of osteoblastic OS, the most common type of OS, our study demonstrated for the first time that CD44 promotes OS growth and dissemination in a HA-dependent manner and that CD44 expression is associated with *in vitro* enhanced migration rates and chemoresistance to cisplatin. This is also consistent with patient's outcome as observed with tissue microarray analysis. On the other hand, in a subgroup of OS, where Ras signaling is increased, CD44 may act as a tumor suppressor probably by upregulation of merlin.

Taken together, the findings presented in this thesis underscore the important role of CD44s/HA interaction in determining tumor malignancy in experimental OS. In conclusion,

our results highlight CD44/HA interaction as a promising target for therapeutic intervention in this highly aggressive cancer type.

2 Zusammenfassung

Das Osteosarkom (OS) ist ein aggressiver Knochentumor, der vor allem bei Kindern und Heranwachsenden auftritt. Im Falle eines lokal begrenzten Tumors haben verbesserte Operationstechniken und chemotherapeutische Behandlung in den letzten paar Jahrzenten zu einem Anstieg der Lebenserwartung der betroffenen Patienten geführt. Bei Patienten, die bereits Metastasen vorweisen, vorwiegend in der Lunge und am Skelett, bleibt die Lebenserwartung weiterhin tief und liegt bei <20%. Um die Lebenserwartung auch bei diesen Patienten anheben zu können, ist es daher wichtig, Einblick zu gewinnen in die molekularen Veränderungen, die zu einem erhöhten Metastasierungspotential und/oder zur Bildung einer Resistenz gegen die Chemotherapeutika führen. Mit der Identifizierung solcher molekularen Komponenten sollte es möglich sein, eine Voraussage machen zu können, welche Patienten einem besonders hohen Risiko ausgesetzt sind und dann für diese Patienten gezielte Therapien zu entwickeln.

Eine Krebszelle muss viele Hindernisse überwinden, um an einem vom Tumor entfernten Ort eine Metastase bilden zu können. Zuerst muss sie sich von der Tumormasse ablösen, dann durch das den Tumor umgebende Gewebe wandern und in ein Lymph- oder Blutgefäss eindringen. Dort muss die Zelle überleben bis sie sich in den Gefässen des Zielorgans anheften muss, um diese wiederum penetrieren zu können. Im Zielorgan angekommen, muss sich die Zelle an die neue Umgebung anpassen und fort geeignete Bedingungen schaffen, um letztendlich erfolgreich das Zielorgan besiedeln zu können. Bei andern Tumorarten als dem OS konnte gezeigt werden, dass einige dieser kritischen Schritte durch das Zelloberflächenprotein CD44 begünstigt werden. CD44 kann Gewebe spezifisch in verschieden langen Formen gebildet werden und dient der Zell-Zell und Zell-Matrix Interaktion. Im Knochen ist ein Hauptbestandteil der extrazellulären Matrix das Hyaluronan (HA), an das die Zellen durch den CD44 Zelloberflächenrezeptor binden können.

In der vorliegenden Arbeit habe ich folgende Aspekte untersucht: 1) Korreliert die CD44 Expression in Gewebeproben von OS Patienten mit deren Ueberlebensrate? 2) Hat die Manipulation der CD44 Expression in OS Zellen einen Einfluss auf deren *in vitro* Eigenschaften? 3) Welchen Einfluss hat die CD44 Expression auf das Metastasierungspotential von OS Zellen nach Transplantation in den Knochen von Mäusen *in vivo*.

Die Expressionsanalyse der OS Gewebeproben zeigte, dass die Ueberlebensrate von Patienten mit Metastasen oder Chemotherapieresistenz zusätzlich erniedrigt ist, wenn die CD44 Expression hoch ist. Als Folge sind diese Patienten mit hoher CD44 Expression einem besonders hohen Risiko ausgesetzt.

In vitro konnte eine positive Korrelation der CD44 Expression mit der Bindung an HA und mit der Migrationsrate der Zellen gezeigt werden. OS Zellen mit erhöhter CD44 Expression waren zudem weniger empfindlich gegenüber Zytostatika.

In vivo konnte gezeigt werden, dass CD44 in osteoblastischen OS Zellen, dem häufigsten OS Typ, beiträgt zu einem vermehrten Tumorwachstum und zu einem erhöhten Metastasierungspotential, was zur *in vitro* beobachteten erhöhten Zellmigrationsrate und Chemoresistenz und zur erniedrigten Ueberlebensrate der Patienten mit erhöhter CD44 Expression passt. In einer Untergruppe von OS allerdings, die eine erhöhte Ras Aktivierung aufweist, scheint CD44 die Tumorbildung und die Metastasierung zu unterdrücken, indem es die Expression des Tumorsuppressors Merlin kontrolliert. CD44 kann dementsprechend Zell spezifisch unterschiedliche Wirkung haben auf die OS Entwicklung.

Die Resultate dieser Studie weisen auf eine wichtige Rolle von CD44 und der Interaktion mit HA bei der Tumorbildung und der Metastasierung beim OS hin. Abhängig

vom Tumortyp können CD44 und/oder HA als therapeutische Ziele ins Auge gefasst werden, um diesen aggressiven Tumor besser behandeln zu können.

3 Introduction

3.1 Osteosarcoma

3.1.1 Definition and epidemiology

Osteosarcoma (OS) is the most common primary tumor of bone in children and adolescents, which is characterized by the presence of malignant spindle cells that produce osteoid and/or immature bone (Picci, 2007). The incidence of OS in the human population is 3 cases per million per year, but amounts to 8-11 cases/million/year in adolescents of between 15 and 19 years of age (Ritter and Bielack, 2010). OS is a disease with bimodal age distribution, with the first peak during the second decade of life, throughout the growth spurt, and the second peak in the elderly adults (Marina et al., 2004). Males are affected more frequently than females (Stiller et al., 2001).

3.1.2 Etiology

The etiology and pathogenesis of OS remains obscure. The only proven exogenous risk factor for developing OS in humans is exposure to ionizing radiation, which accounts for in only 2% of osteosarcomas. Since a long interval of 10-20 years between radiation exposure and OS formation has been observed, radiation-induced OS is typically observed in adults and is not thought to play a major role in pediatric disease. The majority of osteosarcomas are sporadic. However, a number of inherited genetic predispositions have been reported to be associated with OS, which include hereditary retinoblastoma (Rb gene mutation), Li-Fraumeni (p53 mutation), Bloom (BML mutation), Rothmund-Thomson (RECQL4 mutation) and Werner (RECQL2 mutation) syndromes and Paget's disease (SQSTM1 mutation) (reviewed in (Clark et al., 2008; Fuchs and Pritchard, 2002; Wang, 2005).

3.1.3 Cytogenetic and molecular aberrations in OS

Disease-specific chromosomal translocations are characteristic for several sarcomas, such as Ewing's sarcoma and alveolar rhabdomyosarcoma. In contrast, osteosarcomas do not display such specific genetic alterations, but they have a very complex karyotype, with numerical and structural chromosomal abnormalities with considerable heterogeneity (Bridge et al., 1997; Helman and Meltzer, 2003).

3.1.4 Clinical characteristics and diagnostics

The typical symptoms of OS are local pain, followed by soft tissue swelling, which usually arise after trauma or vigorous physical training (Marina et al., 2004). In rare cases, patients present with pathological fractures. Although OS can occur in any bone, it is mainly observed in the metaphysis of long bones. The most common primary sites are the distal femur (40%), the proximal tibia (20%) and the proximal humerus (10%), with approximately 50% of the cases occurring around the knee (Longhi et al., 2006). OS rarely develops in the axial skeleton (<10% of patients in the pediatric age), where it mainly affects the pelvis (Ritter and Bielack, 2010).

At the time of diagnosis, between 15 and 20% of the patients present with detectable metastases. However, 80% of the patients with localized disease develop metastases after surgical resection. The metastases are commonly found in the lungs and less frequently in the bone. The major cause of death in OS patients are pulmonary metastases with respiratory failure due to widespread disease (Marina et al., 2004).

Clinical examinations for the diagnosis of OS comprises full patient history, physical examination, plain radiography, magnetic resonance imaging (MRI), computed tomography (CT), dynamic bone scintigraphy and biopsy. Plain radiography of bone affected by OS shows characteristic osteoblastic, osteolytic or mixed lesions. Ossifications in the soft tissue often

appears as a radial, so called “sunburst” pattern. Codman’s triangle is a specific feature seen in radiographs that results from periostal new bone formation and lifting of the cortex (Figure 1).

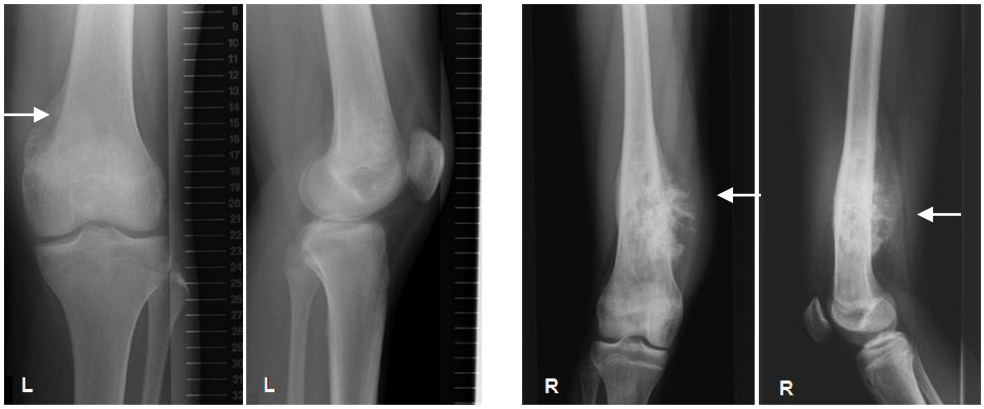


Figure 1: Typical radiographic appearance of OS. Anteroposterior (**A**) and lateral (**B**) radiograph of the left distal femur show a typical “Codman`s triangle” (arrow) in an OS patient. A “sunburst”-type periostal reaction (arrows) can be seen in the anteroposterior (**C**) and lateral (**D**) radiograph of the right distal femur of an OS patient.

CT and MRI are used to assess the extensions of the tumor and the involvement of surrounding structures such as vessels, nerves and soft tissue (Aisen et al., 1986). Chest CT and isotope scans with technetium or thallium are applied in order to visualize lung and bone metastases (Figure 2) (Wittig et al., 2002).

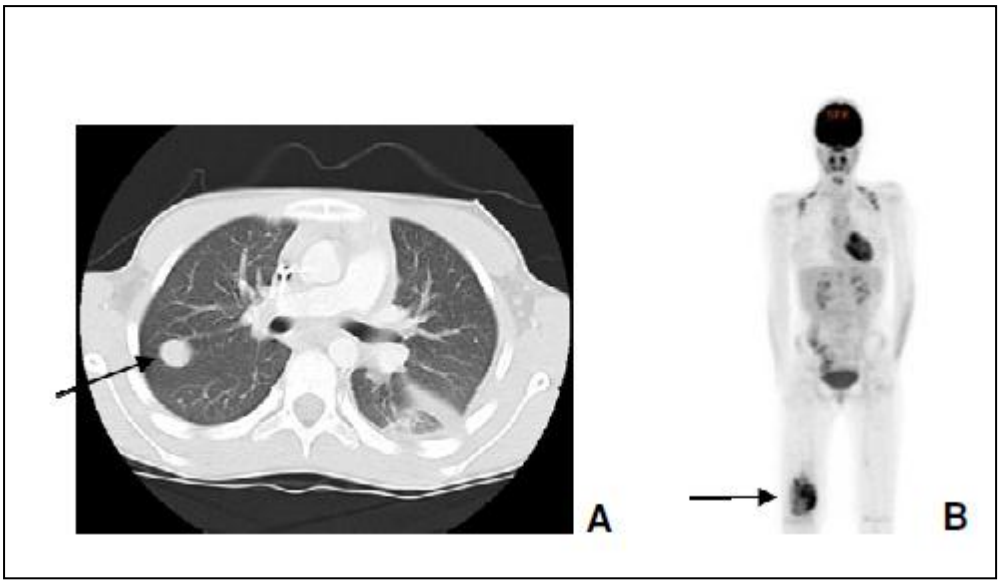


Figure 2: CT and scintigraphic imaging of OS. (**A**) Chest CT scan indicating a large lung metastasis (arrow). (**B**) Bone scintigraphy corresponding to the X-ray shown in Figure 1c, d. High uptake of ^{99m}Tc in the right distal femur (arrow). High uptake in the bladder, kidney, heart and brain are regular findings.

Diagnostic histology of primary tumor biopsy tissue is mandatory to confirm the radiological findings. OS has a wide spectrum of histological appearances, but the hallmark is the presence of osteoid or immature bone produced by malignant mesenchymal tumor cells (Longhi et al., 2006). Three major subtypes of conventional OS are recognized by the World Health Organization (WHO): osteoblastic, chondroblastic and fibroblastic OS, classified based on the predominant type of matrix within the primary tumor. Additional histologic variants include teleangiectatic, small cell, parosteal, periosteal, low grade central and high grade surface OS (Marina et al., 2004).

The most widely used staging system, the Enneking system, combines histologic grading (low grade: stage I; high grade: stage II) and anatomical tumor extension (A: intracompartmental; B: extracompartmental). Patients with distant metastases are categorized as stage III. The vast majority of cases are stage IIB at presentation (Enneking et al., 1980).

3.1.5 Treatment

Current state-of-the-art treatment of OS patients requires a multidisciplinary approach and includes neoadjuvant (preoperative) multi-agent chemotherapy, followed by resection of the primary tumor and subsequent adjuvant (postoperative) chemotherapy (Ta et al., 2009). Complete surgical resection of the primary tumor is fundamental for OS cure. Traditionally, amputation was the sole treatment, however, advances in surgical techniques enabled the limb-sparing procedures, which are nowadays safely performed in 90%-95% of the patients (Wittig et al., 2002). Neoadjuvant and adjuvant therapies, introduced in the late 1970's with the goal to destroy tumor cells, to decrease the tumor burden and to eradicate micrometastases, resulted in a significant increase in the 5-year disease-free survival to up to 70% (Bacci et al., 2002; Campanacci et al., 1981; de Kraker and Voute, 1989; Enneking, 1979; Rosen et al., 1982; Rosenburg et al., 1979). Doxorubicin, cisplatin, ifosfamide and high-dose methotrexate have been shown to be most effective in OS treatment. However, polychemotherapy may be associated with severe toxicities, such as permanent damage of cardiac (Lipshultz et al., 1995), renal (Rossi et al., 1994), auditory and reproductive function and other late effects including secondary malignancies (Aung et al., 2002). The mechanisms of action and major side effects of current chemotherapeutics used are listed in Table 1.

Agent	Mechanism of action	Side effects
Doxorubicin (Adriamycin)	Inhibition of DNA and RNA synthesis by intercalating at points of local uncoiling of the DNA double helix.	Cardiomyopathy, transient electrocardiographic abnormalities, emesis, alopecia, mucositis, myelosuppression
Cisplatin (Platinol)	Inhibition of DNA synthesis through the formation of DNA cross-links	Acute or chronic renal failure, peripheral neuropathy, ototoxicity, emesis, myelosupression, alopecia, hypomagnesemia
Ifosfamide	Inhibition of DNA synthesis by crosslinking DNA strands	Hemorrhagic cystitis, renal failure, myelosuppression, alopecia, emesis, encephalopathy
Methotrexate	It is a antifolate,, and inhibits synthesis of purine and thymidine by binding to dihydrofolate reductase	Renal failure, mucositis, mild myelosuppression, rarely, central nervous system effects

Table 1. Chemotherapeutic agents for the treatment of OS

The assessment of tumor necrosis following preoperative therapy has been proven to be a reliable prognostic factor and it correlates with disease-free and overall survival (Bielack et al., 2002; Glasser et al., 1992). The degree of necrosis is evaluated according to the Huvos grading system (Huvos, 1981)(Bielack, Kempf-Bielack et al. 2002). The patients are classified as poor responders if they show <90% of necrosis (Grade I: no necrosis, Grade II: 50%-90% of necrosis), whereas good responders correspond to Grade III (necrosis between 90% and 100%) and Grade IV (total necrosis) (Bielack et al., 2002) . The histological response to neoadjuvant therapy correlates significantly with prognosis and allows tailoring of

the postoperative treatment according to different risk groups. Currently, a randomized trial of the EURAMOS (European and American Osteosarcoma; Figure 3) study group aims at optimizing the treatment modality for individual patients based on response to preoperative chemotherapy (Bielack et al., 2008; Carrle and Bielack, 2009).

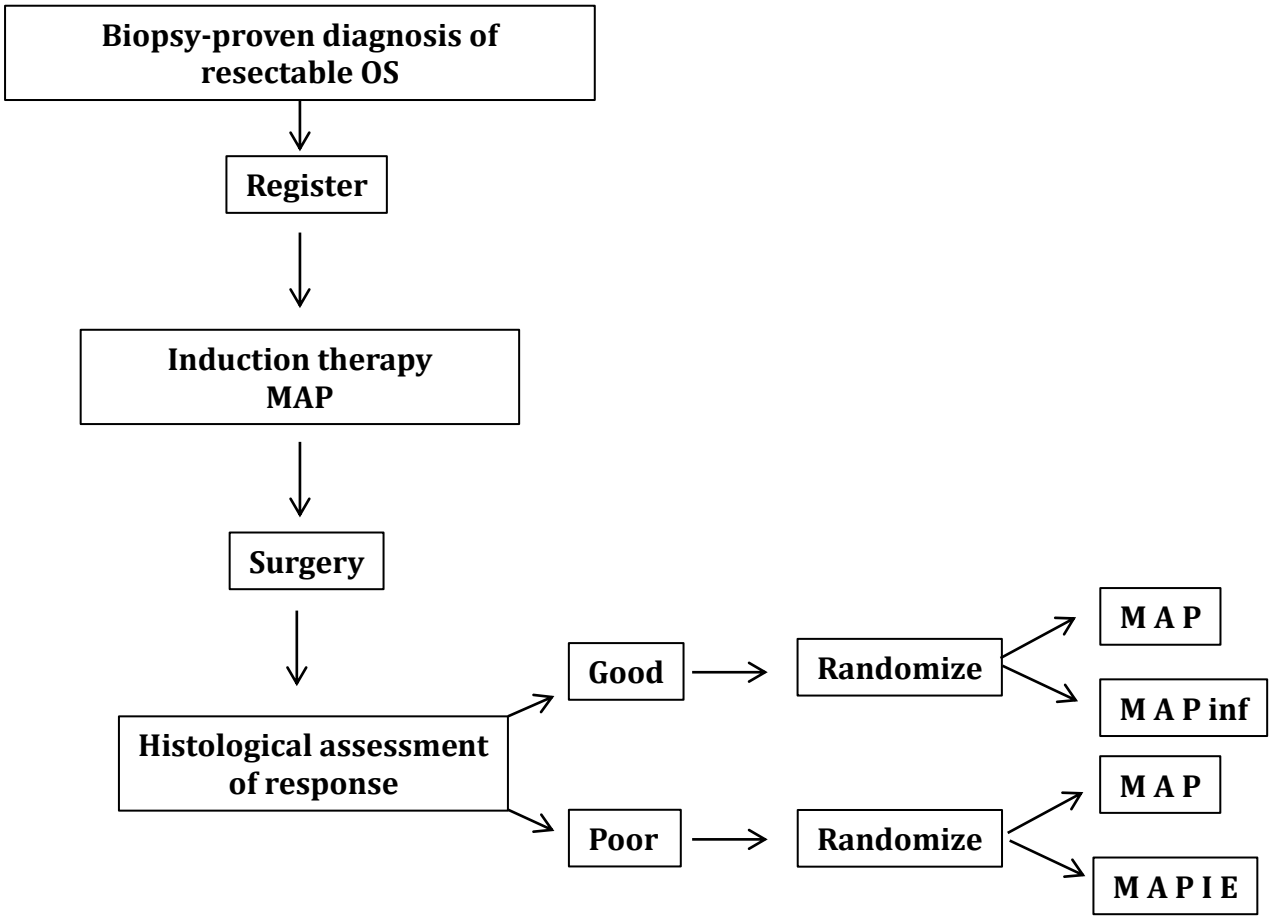


Figure 3: EURAMOS 1 treatment outline. OS patients receive 2 cycles of neoadjuvant chemotherapy with doxorubicin, cisplatin and methotrexate followed by surgery. Subsequently they are divided into 2 groups distinguishing poor responders from good responders according to Huvos' classification. Good responders are randomly subdivided into 2 groups, with 1 group additionally receiving interferon-alpha as maintenance therapy after 4 cycles of adjuvant chemotherapy. Poor responders are also randomly subdivided in 2 groups to evaluate the benefit of etoposide/ifosfamide amendment. M = high-dose MTX, A = adriamycin (doxorubicin), P = Cisplatin, ifn = pegylated interferon alpha maintenance, I = high-dose ifosfamide, E = etoposide (Adapted from (Bielack et al., 2008)).

These significant clinical improvements over the past several decades through the use of combined chemotherapy and surgery have led to a dramatic increase in the survival of patients with localized disease. However, patients with metastatic or recurrent disease continue to have a poor prognosis, with <20% long term survival (Marina et al., 2004). Therefore, it is of great importance to elucidate the molecular mechanisms leading to increased metastatic potential. Recently, a number of emerging molecular markers have been shown to play an important role in OS metastasis. The identified genes are mostly involved in cell adhesion, migration and angiogenesis. Some examples that have been correlated to prognosis are ezrin, MMP-2, MMP-9, CXCR4/SDF1, uPA/uPAR and survivin. Several of them are already being tested as potential therapy targets in preclinical trials (reviewed in (Clark et al., 2008; Wang, 2005)).

In conclusion, a better understanding of the basic biology of OS progression will enable us to devise novel strategies aiming at suppression of metastasis, the major cause of death in OS, and to finally improve the survival of these patients.

3.2 Metastasis

One of the hallmarks of malignant tumors is their ability to metastasize and >90% of deaths in cancer patients is attributable to metastatic disease. Metastases remain the main barrier to successful cancer management because of their systemic nature and resistance to conventional therapies. The gain of metastatic ability of most cancers leads to clinically incurable disease. Metastasis is a complex multistep process, often referred to as "invasion-metastasis cascade", which involves spread of cancer cells from their primary site and establishment of new colonies in anatomically distant organs (Valastyan and Weinberg, 2011).

The metastatic process includes detachment of tumor cells from primary tumor mass, invasion of local stroma, intravasation (penetration of local blood and/or lymphatic vessels), survival during transport in the circulation, arrest at distant organ sites, extravasation into corresponding parenchyma, adaptation to this new foreign tissue microenvironment and finally reinitiation of proliferation of micrometastatic lesions and generation of macroscopic metastases, defined as colonization. In order to give rise to a metastatic tumor, a tumor cell needs to go through all these processes successfully. Particular stages, such as colonization, are highly inefficient and, consequently, rate limiting (Figure 4). Efficient steps in the metastatic cascade, on the other hand, include survival in the circulation, arrest at distant sites and extravasation. Experimentally it was shown that a tiny proportion (<0.02%) of intravenously injected cells generate macroscopic metastases (Luzzi et al., 1998). The final outcome of the metastatic process – the formation of clinically relevant lesions - is governed both by the intrinsic properties of cancer cells and their multiple and complex interplay with host components (Chambers et al., 2002; Fidler, 2003; Langley and Fidler, 2007).

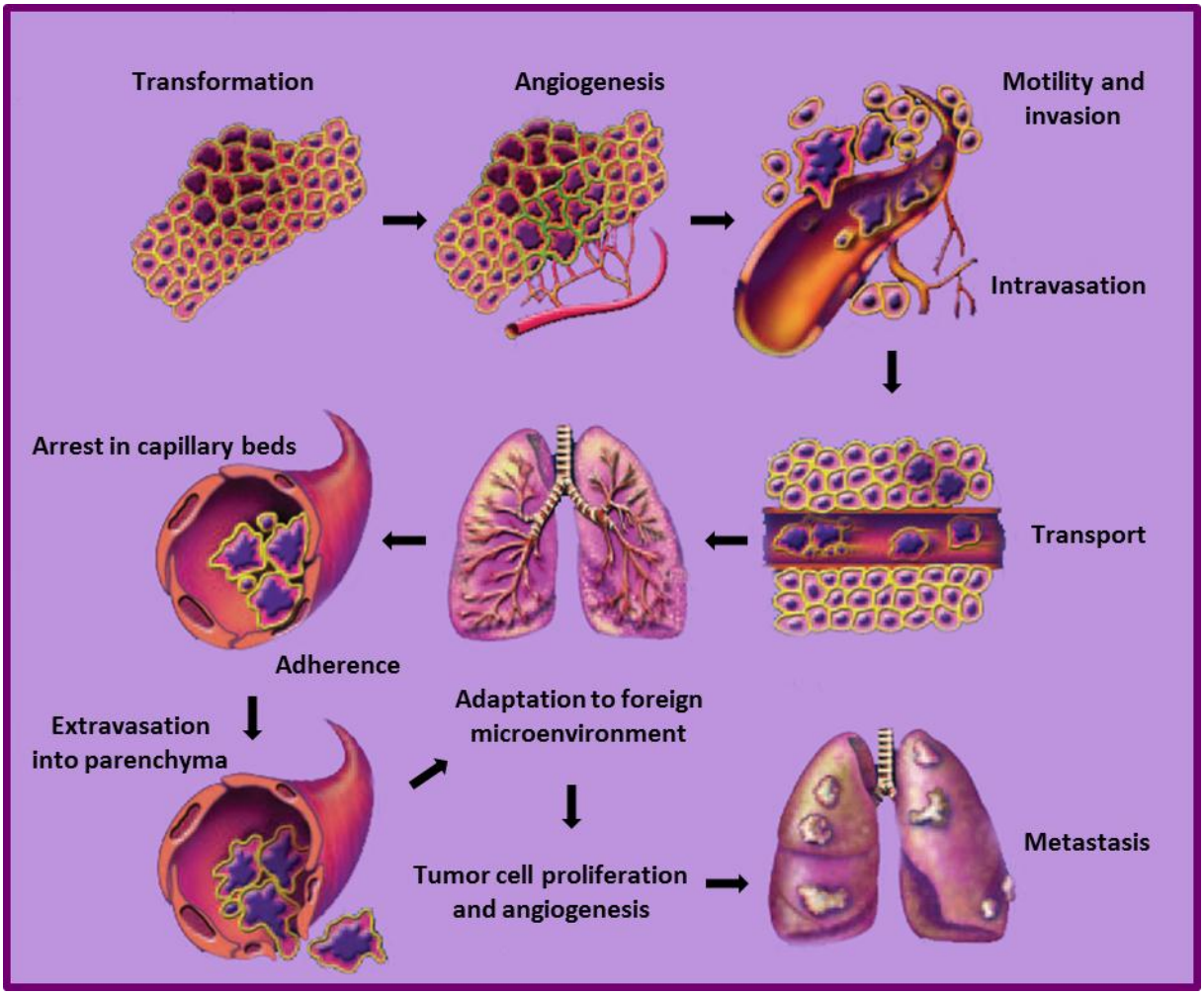


Figure 4: The invasion-metastatasis cascade. The metastatic process is depicted as a sequence of interlinked, distinct steps. Tumor cells escape the primary tumor site through local invasion of surrounding stroma, migration and intravasation of lymphatic and/or blood vessels. Once in the circulation, cancer cells must avoid the immune attack and survive the transport until they are trapped in capillary beds of a distant organ. The arrest of circulating tumor cells at a specific site may occur by mechanical entrapment due to size restriction and by specific adhesive interactions. Following extravasation, tumor cells must adapt to the foreign milieu. Some micrometastases will eventually acquire the ability to reinitiate growth and form macroscopic metastases. The last step in the cascade – colonization - is the most inefficient of all. (Adapted from (Langley and Fidler, 2007)).

3.2.1 Organ-specific metastasis

Clinical observation of cancer patients revealed that certain tumor types have a propensity to metastasize to preferred distant organs (Figure 5). For instance, breast cancer usually spreads to bone, liver, brain and lungs, whereas prostate cancer metastasizes preferentially to bone. Metastases of colorectal cancer occur most prominently in the liver. Pulmonary metastases frequently develop in patients suffering from osteosarcoma.

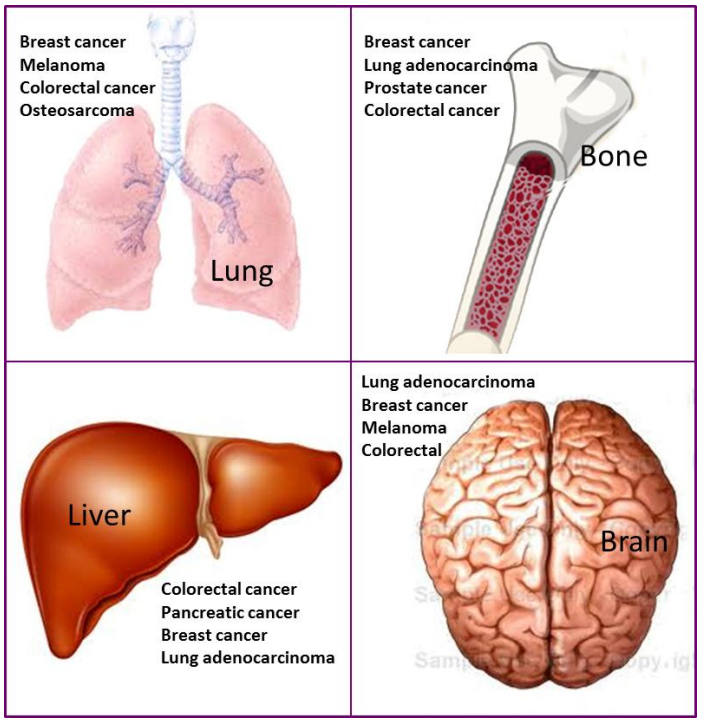


Figure 5: Characteristic sites of metastatic spread for solid tumors.
(Adapted from (Chiang and Massague, 2008)).

In 1889, Stephen Paget published the "seed and soil" hypothesis of metastatic outgrowth, whereby he explained the non-random pattern of metastatic colonization, proposing that metastasis depends on the cross-talk between cancer cells ("seed)" and the microenvironment within specific distant organs ("soil") (Paget, 1989). In 1928, James Ewing challenged Paget's theory, claiming that anatomical arrangement of the vascular system determines the organ-specific metastasis (Ewing, 1928). The "seed and soil" theory was revived when studies by Isaiah Josh Fidler demonstrated that, although the cancer cells reached the vasculature of all organs, metastatic lesions selectively developed in certain organs, but not others (Fidler, 2003; Fidler and Kripke, 1977; Hart and Fidler, 1980; Psaila and Lyden, 2009). Eventually, both concepts revealed the proposal that the dissemination of tumor cells is affected by circulatory routes, but in fact the outgrowth of macrometastasis depends on the seeding of compatible tissues.

The tissue tropism of metastasizing cells to specific sites can be explained by several mechanisms: chemokine-receptor mediated chemotaxis, the establishment of a metastatic niche, and a genetic program within tumor cells that enables them to adapt to a particular microenvironment (Bacac and Stamenkovic, 2008).

The first mechanism involves chemokines and their receptors. Target organs release chemoattractants that actively guide tumor cells expressing "matching" chemokine receptors to specific destinations from the circulation (Joyce and Pollard, 2009; Muller et al., 2001; Zlotnik, 2004). For example, breast cancer cell express high levels of CXCR4 and CCR7. Signaling through these chemokine receptors mediates actin polymerization and pseudopodia formation that contributes to a chemotactic and invasive response. The homing is achieved through respective cognate ligands CXCL12 and CCL21, which are expressed in lymph nodes, lung, liver and bone marrow tissue - sites to which breast tumors commonly metastasize - but not in other organs. Moreover, blocking of either CXCL12 or CXCR4 inhibits metastasis of breast cancer cells in animal models (Muller et al., 2001). The involvement of the CXCL12/CXCR4 axis has also been implicated in OS metastasis progression (Perissinotto et al., 2005).

The metastatic niche model proposes that primary tumors produce factors that orchestrate the preparation of the local parenchyma in distant organs prior to the seeding of metastatic cells. Cancer cells and their associated stromal cells secrete an array of chemokines that recruit bone-marrow derived endothelial and hematopoietic progenitor cells to relevant organs prior to tumor cell arrival. These cells expressing VEGFR1 were suggested to alter the microenvironment, which further promotes homing and engraftment of circulating tumor cells in these specific destination sites (Kaplan et al., 2006; Kaplan et al., 2005).

Gene expression profiling of breast cancer primary tumor tissue revealed a “metastasis signature” that predicts onset of metastasis and correlates with poor prognosis of the patients (Ramaswamy et al., 2003; van 't Veer et al., 2002). It has also been implied that a set of genes may regulate organ specific metastatic dissemination. Minn et al. identified a set of genes responsible for the lung tropism of the human MDA-MB231 breast carcinoma cell line (Minn et al., 2005). The same cell line has been used to identify genes that mediate breast cancer metastasis to bone (Kang et al., 2003).

3.2.2 Metastasis genes

Three classes of genes controlling the biological processes during the metastatic cascade have been defined:

- metastasis initiation genes
- metastasis progression genes
- metastasis virulence genes (Nguyen et al., 2009).

Metastasis initiation genes promote cell motility, epithelial-mesenchymal transition (EMT), degradation of the extracellular matrix (ECM), invasion, recruitment of bone marrow progenitors, angiogenesis or evasion of the immune system. For example, EMT programs are orchestrated by a set of pleiotropically acting transcription factors, such as TWIST1, SNAI1 and SLUG (Yang and Weinberg, 2008). Invasion is supported by metadherin in breast cancer (Hu et al., 2009) and by the metastasis-associated in colon cancer 1 (MACC1) gene in colorectal carcinoma (Stein et al., 2009).

Metastasis progression genes can provide both local advantage in the primary tumor and distal advantage in the metastatic microenvironment. These genes regulate extravasation, survival in the circulation and re-initiation of proliferation at the distal site. MMP1, MMP2, EREG, ANGPTL4, COX2 belong to this group of genes and were shown to enhance the

extravasation of breast cancer cells in the lungs, by disrupting cell-cell junctions between pulmonary vascular endothelial cells (Gupta et al., 2007; Padua et al., 2008).

Metastasis virulence genes, however, don't participate in primary tumor development, but are essential for organ-specific metastatic colonization, the final step of the invasion-metastatic cascade. Examples of this group of genes are interleukin 11 (IL11) and parathyroid hormone-related protein (PTHRP), which facilitate the formation of osteolytic metastasis in the bone by breast tumor cells, but do not provide advantage in primary tumors (Mundy, 2002; Yin et al., 1999).

3.2.3 Adhesion-molecular effector mechanism of metastasis

The relevance of cell-cell and cell-matrix adhesion and their involvement in all the subsequent phases of the metastatic journey has been well established. In addition to proteolysis, adhesion is considered a fundamental molecular effector mechanism employed by a metastatic cell (Bacac and Stamenkovic, 2008).

Adhesion molecules can modulate metastasis both in a positive and a negative manner. Detachment of cancer cells from the primary tumor mass and infiltration of adjacent tissue is supported by loss of intercellular contacts through downregulation of cell surface adhesion molecules. Continued changes in the adhesive properties of tumor cells facilitate their locomotion on vessel endothelium and trans-endothelial migration, survival in the blood or lymphatic stream, movement through host parenchymal tissues and finally restart of growth at secondary sites.

In conclusion, successful execution of metastatic dissemination requires tumor cells to display major phenotypic plasticity, which enables them to appropriately interact with surrounding cells and matrix elements at each stage of the metastatic process. Adaptation of a cells' adhesion profile is substantially dependent on the activity of different classes of cell

adhesion molecules, such as cadherins, integrins, members of the immunoglobulin superfamily and CD44 (Balzer and Konstantopoulos, 2011).

3.3 CD44

The CD44 protein family consisits of widely expressed type I transmembrane glycoproteins with a large repertoire of biological functions in both health and disease. They participate in various physiological and pathological processes including development, wound healing, inflammation, hematopoiesis, immune response and tumor progression. CD44 molecules are the principal receptors for hyaluronan (HA), a major component of the extracellular matrix also in bone (Aruffo et al., 1990). The functional diversity is due to the structural heterogeneity of CD44 glycoproteins, resulting from alternative splicing of initial gene transcripts and post-translational modification of the proteins. CD44 glycoproteins can vary in molecular weight from 80 to 200 kDa (Herrlich et al., 1998; Naor et al., 1997; Ponta et al., 2003).

3.3.1 CD44 gene and protein structure

All CD44 isoforms are encoded by a single gene, located in humans on chromosome 11 (Goodfellow et al., 1982) and in mice on chromosome 2 (Colombatti et al., 1982). The gene consists of 20 exons, 10 of which can be subject to alternative splicing (variant v1-v10 exons) (Figure 6) (Screaton et al., 1992). Theoretically, multiple combinations of variant exons could give rise to more than 1000 different isoforms. Tissue-specific splicing results in the formation of the standard CD44 isoform (CD44s), lacking all variant exons, in cells of mesenchymal origin (Figure 6). It is ubiquitously expressed and located in the membrane of most vertebrate cells during fetal development and in adult organisms. The variant isoforms, named by the exons they contain, are expressed in only few epithelial tissues, mainly in proliferating cells, during embryonic development and in several cancers (Naor et al., 1997).

Some differentiated ectodermal cells, on the other hand, express an isoform that contains all the protein domains encoded by the variant exons (Hudson et al., 1995; Sherman et al., 1998). Interestingly, alternative splicing is also controlled by mitogenic signals, such as the stimulation of the Ras-Mek-Erk pathway (reviewed in (Marhaba and Zoller, 2004)).

All CD44 proteins structurally comprise a large extracellular domain, a transmembrane region and an intracellular cytoplasmic tail (Figure 7). The extracellular region includes an amino-terminal globular domain, encoded by exons 1 to 5, and the membrane-proximal stem structure (stalk region), encoded by the exons 16 and 17. The variant exon products can be incorporated in this stem structure. Exon 18 encodes the transmembrane domain, whereas the intracellular cytoplasmic region is encoded by the exons 19 and 20. A very rarely expressed short version of CD44 contains a cytoplasmatic tail of only 3 amino acids (Goldstein and Butcher, 1990).

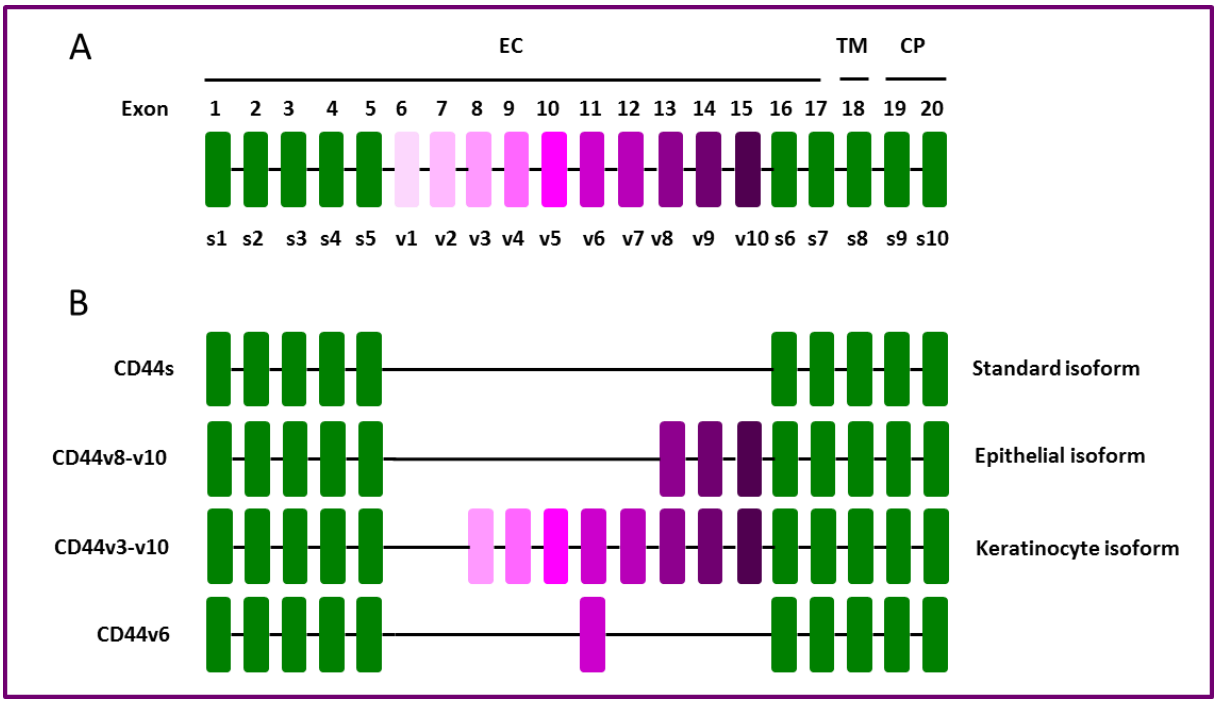


Figure 6: CD44 gene structure. (**A**) CD44 exon map. Green bars represent exons encoding constant regions, colored bars indicate variant exons inserted into final transcripts by alternative splicing. EC, extracellular domain, TM, transmembrane domain, CP, cytoplasmatic domain. (**B**) Examples of variant CD44 isoforms. (Adapted from (Zoller, 2011)).

The amino-terminal globular structure is responsible for HA binding through two binding sites: the link domain (amino acids 32-132) and a basic motif outside the link domain (amino acids 150-158) (Aruffo et al., 1990; Screaton et al., 1992). The amino-terminal domain can also interact with several additional molecules including other glycosaminoglycans (GAGs), laminin, fibronectin and collagen (Naor et al., 1997). It is extensively modified by N- and O-linked glycosylation, which influences the affinity for the different CD44 ligands. With the insertion of variant exon products new sites for secondary modification are introduced. For instance, sequence encoded by exon v3 has sites for modification with chondroitin sulfate or heparin-sulfate to which several heparin-binding proteins such as fibroblast growth factor 2 (FGF2) can bind (Bennett et al., 1995). CD44v6 contains a binding site for hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) (Orian-Rousseau and Ponta, 2008; Tremmel et al., 2009). Moreover, CD44 isoforms containing the sequences encoded by the exons v6 and v7 interact with osteopontin (Katagiri et al., 1999). CD44 can also bind through its GAG binding sites to proteoglycans, such as versican (Kawashima et al., 2000), aggrecan (Fujimoto et al., 2001), and serglycin (Toyama-Sorimachi et al., 1995). However, the functional relevance of these interactions still needs to be clarified.

Dynamic regulation of the interaction between CD44 and the extracellular matrix during cell migration is brought about shedding of the ectodomain of CD44 by proteolytic cleavage within the stem structure (Okamoto et al., 1999ba). It is triggered by multiple signals including the influx of extracellular Ca^{2+}, the activation of protein kinase C (PKC), Rho family of small GTPases, Rac and Ras oncoproteins (Okamoto et al., 1999ab). This cleavage is mediated by matrix metalloproteinases (MMPs), such as MT1-MMP, ADAM10 and ADAM17.

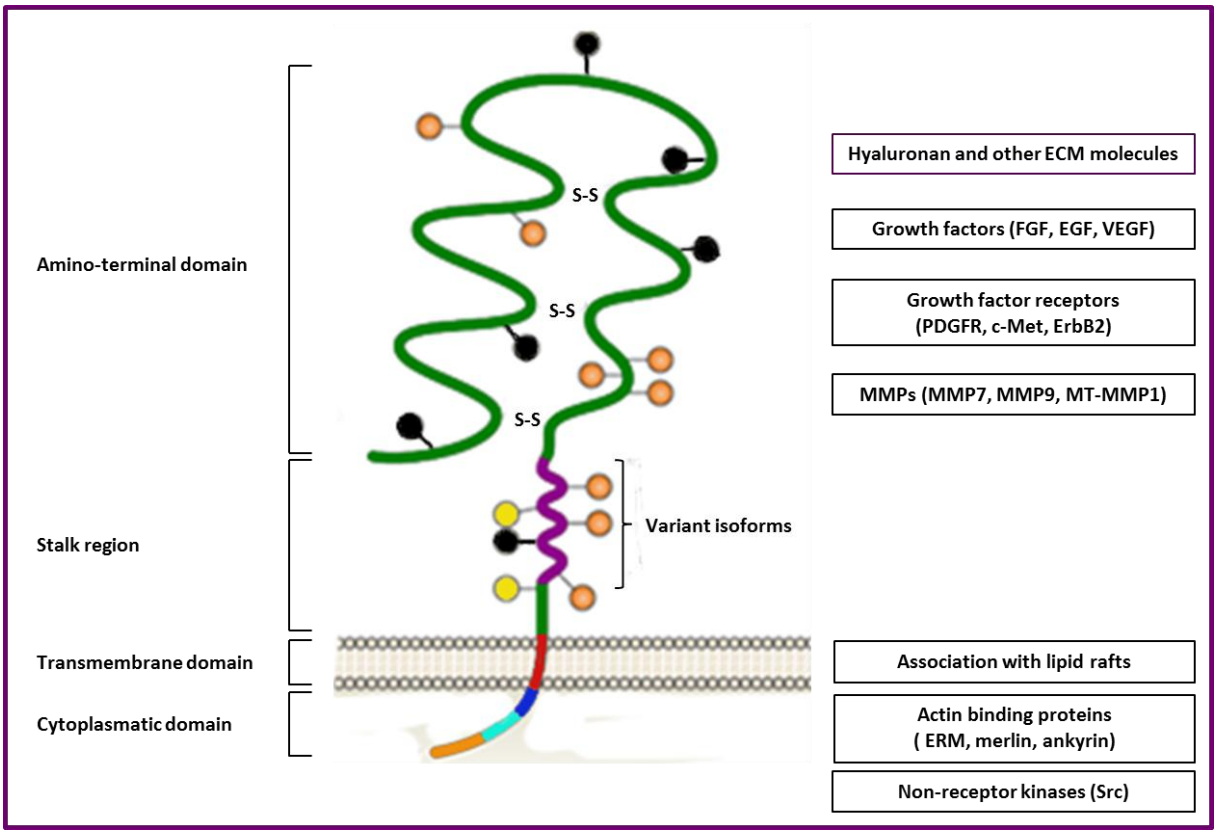


Figure 7: Protein structure and interactions of CD44. The CD44 protein consists of an extracellular, a single transmembrane and a cytoplasmatic domain. The extracellular domain includes the N-terminal - and the stalk region close to the transmembrane domain. This region varies in different isoform due to splicing of the variant exons in different combinations in a tissue specific manner. The ectodomain contains binding sites for HA enables its interaction with growth factors, growth factor receptors and matrix metalloproteinases. Multiple sites for N-glycosylation (black circles) and O-glycosylation (orange circles**)** exist in this domain. There are two GAG binding sites (yellow circles), one of which is located in a CD44 domain encoded by exon v3. The cytoplasmatic tail includes the ERM (dark blue) and ankyrin-binding (light blue) domains, as well as binding motives for Src phosphokinases lck, lyn and fyn. S-S, disulphide bonds. (Adapted from (Misra et al., 2011; Zoller, 2011)).

The transmembrane domains of individual CD44 molecules associate with each other in oligomers and with lipid rafts (Neame et al., 1995; Perschl et al., 1995).

Interactions of the C-terminal cytoplasmatic tail with ankyrin and members of the ERM (ezrin, radixin and moesin) family of proteins link CD44 with the cytoskeleton (Kalomiris and Bourguignon, 1989; Tsukita et al., 1994). Phosphorylation of serine residues in this domain, mediated by protein kinase C, modulates interactions of CD44 with ERM proteins. Phosphorylation of Ser-291 abrogates ezrin binding and modulates CD44-mediated directional cell motility (Legg et al., 2002). Merlin, a tumor suppressor structurally related to

the ERM family, also associates with the cytoplasmic tail of CD44. Dephosphorylation of merlin, which is associated with its growth inhibitory function, is provoked by high cell density or addition of high-molecular weight HA and depends on its interaction with the cytoplasmatic tail of CD44 (Morrison et al., 2001; Shaw et al., 1998). Moreover, intracellular signaling molecules, such as kinases Src, LCK, Fyn, Rho GTPase, Rho kinase and the nucleotide exchange factors TIAM1 and VAV2, also associate with the intracellular C-terminal domain of CD44 (Naor et al., 1997; Ponta et al., 2003), but the nature and the functional relevance of these interactions are not yet understood in detail.

3.3.2 CD44 in cancer

CD44 has been reported to be essential for many tumor activities and its expression in tumor tissue was shown to be associated with increased metastatic spread in different types of cancer. Over the last decade, numerous studies have addressed the relevance of CD44 isoforms as prognostic factors for human cancers. The results suggested that, depending on the affected organ, the pattern of CD44 isoforms expression and the occurrence of metastases are directly or inversely related. The expression of various CD44 isoforms was found upregulated in many human tumor types, including gastric cancer, pancreatic cancer, lung and renal cell cancer (Heider et al., 1993; Lim et al., 2008; Penno et al., 1994; Rall and Rustgi, 1995). The results of several reports point to important functions of the standard form of CD44 (CD44s) in tumor progression (Cannistra et al., 1993). However, in other tumor types as neuroblastoma and prostate cancer, the lack of CD44 expression (both the standard isoform and the splice variants) indicated poor prognosis (Angelo M. De Marzo, 1998; Shtivelman and Bishop, 1991) and CD44 even acts as a metastasis-suppressor gene in prostate carcinoma cells (Gao et al., 1997; Ponta et al., 2003).

3.3.3 CD44 mechanisms of action

Three types of molecular actions underlie the multifunctionality of CD44 (Ponta et al., 2003). First, ectodomains of CD44 molecules on the cell surface act as receptors for ligands of the ECM, predominantly HA, and form "platforms" that orchestrate the assembly of cell surface protein complexes that include matrix metallo-proteinases and growth factors. Second, they are co-receptors of several receptor tyrosine kinases and thereby modulate the signaling of associated growth factor receptors. Third, they act as organizers of the actin cytoskeleton.

Consequently, adhesiveness, motility, matrix degradation, proliferation and cell survival can be modulated by CD44 through interplay with its ligands and associated molecules. CD44 mediated regulation of these cellular processes enables the tumor cells to successfully proceed through all the steps of the metastatic cascade (Marhaba and Zoller, 2004).

3.3.3.1 CD44 as a ligand-binding surface receptor

As outlined before, a predominant property of CD44 is its ability to bind HA. The HA binding sites are located in the standard part of the CD44. Importantly, insertion of variant exon products or the state of glycosylation may influence the affinity of the binding.

Interestingly, not all CD44 expressing cells are capable to bind HA. In many cases, CD44 doesn't bind to HA unless activated by external stimuli. Through this mechanism unnecessary engagement of the receptor is avoided, since both the ligand and the receptor are ubiquitously expressed. CD44 molecules are apparently expressed at the cell surface in three states of variable activity (reviewed in (Naor et al., 2002)):

- Active CD44, which binds HA constitutively;
- Inducible CD44, that binds HA upon activation by different stimuli, including cytokines (interleukin 5, tumor necrosis factor α), growth factors (EGF), oncostatin M and phorbol ester. The binding can also be induced by CD44 crosslinking, which results in either conformational changes or redistribution of CD44 in the plasma membrane;
- Inactive CD44, which is unable to bind to HA, even in the presence of inducing agents.

Despite extensive research, there are still conflicting data regarding the importance of CD44-HA interaction in the regulation of tumor malignancy. CD44 has been shown to promote tumor and metastasis development both in a HA-dependent and HA-independent fashion. Several lines of evidence indicated that CD44 mediated binding of cancer cells to HA-rich ECM triggers cell signaling pathways that regulate the migration, invasion and lodging of tumor cells in distant organs (Misra et al., 2011; Thomas et al., 1992). Yu et al. demonstrated the role of CD44-HA interaction in promoting tumor invasion (Yu et al., 1997). In their study, overexpression of a soluble CD44 ectodomain in murine metastatic mammary carcinoma cells suppressed the binding and internalization of HA by endogenous cell surface-located CD44 and, consequently, the invasion on HA-producing cell monolayers. In an experimental metastasis model in mice, intravenously injected CD44 ectodomain-overexpressing cells, unlike non-transfected control cells, formed only a few or no metastases in the lungs. Both cell types adhered to the pulmonary endothelium and were able to penetrate the interstitial stroma, but the mammary carcinoma cells overexpressing the soluble CD44 ectodomain underwent apoptosis. These data indicate that one of the mechanisms, by which CD44-HA interaction supports the metastatic process, is the inhibition of apoptosis. In a comparable study with a human melanoma cell line, Ahrens et al. demonstrated that

overexpression of soluble HA binding CD44 suppressed subcutaneous primary tumor growth in mice, whereas cells overexpressing HA binding-defective soluble CD44 formed fast growing subcutaneous tumors much like the non-manipulated human melanoma cells (Ahrens et al., 2001). These results implicate that the observed growth inhibition is dependent on the interaction between soluble CD44 and HA, which competes for the interaction of HA with endogenous CD44 on the cell surface.

In contrast, transfection of a pancreatic carcinoma cell line with a CD44v4-v7 isoform conferred metastatic activity (Günthert et al., 1991) independent of HA binding, since removal of HA by degradation had no impact on the metastatic potential of CD44v4-v7 overexpressing cells (Sleeman et al., 1996).

3.3.3.2 CD44 as a "platform" for enzymes and substrates

It has been described that CD44 proteins concentrate MMPs, such as MMP7 (Yu et al., 2002) and MMP9 (Yu and Stamenkovic, 1999), on the cell surface. For example, CD44-HA aggregates facilitated recruitment of MM9 to the cell surface of mouse mammary carcinoma and human melanoma cell lines and thereby promoted degradation of collagen IV and tumor-cell invasion (Yu and Stamenkovic, 1999). Furthermore, in addition to collagen degradation, MMP9 is also responsible for proteolytic activation of TGFβ (tumor growth factor β) that triggers angiogenesis (Yu and Stamenkovic, 2000). Similar to MMPs, a variety of growth factors and cytokines are also immobilized on the cell surface through interaction with CD44. Examples have been described before.

3.3.3.3 CD44 as a co-receptor

An emerging concept in signal transduction nowadays is that cell-adhesion molecules, once believed to primarily act as cell surface molecules attaching cells to extracellular ligands, have additional functions as co-receptors in cellular signaling cascades (Ponta et al., 2003).

Accordingly, the CD44 isoforms containing an exon v6 encoded domain are essential for the activation of c-Met by HGF/SF and was found to form a complex with the ligand and its cognate receptor in a rat and in human carcinoma cell lines, as well as in primary keratinocytes. Furthermore, the cytoplasmatic tail of CD44 is required for further signal transduction from activated c-Met (Orian-Rousseau et al., 2002). A second interaction, relevant in tumor cells, was observed between CD44 and receptor tyrosine kinases of the ERBB receptor family. CD44 co-immunoprecipitates with ERBB1 (EGFR/HER1), ERBB2 (HER2/neu), ERBB3 (HER3) and ERBB4 in several cell lines and primary cells (Bourguignon et al., 1997; Sherman et al., 2000; Yu et al., 2002). Moreover, the heparin-binding epidermal growth factor (HBEGF) preform binds to CD44v3 modified by heparin-sulphate side chains, and it is subsequently cleaved by MMP7, which is recruited to the cell surface by CD44. Cleaved HBEGF then activates its receptor ERBB4 that signals for cell survival (Yu et al., 2002). CD44, on the other hand, also mediates, through direct interaction, the heterodimerization of ERBB2/ERBB3 in response to neuregulin. The resulting receptor activation is crucial for differentiation, survival and proliferation of Schwann cells (Meyer and Birchmeier, 1995; Riethmacher et al., 1997; Sherman et al., 2000).

3.3.3.4 CD44 as an organizer of the cytoskeleton

The interaction of CD44 with the actin cytoskeleton is indirect and is achieved via linker proteins, including ankyrin and the members of the ERM family (Bourguignon et al., 1998; Tsukita et al., 1994). Ankyrin mediates the contact with spectrin and plays a role in HA-dependent cell adhesion and motility (Lokeshwar et al., 1994). ERM proteins are involved in the regulation of cell migration, cell shape determination and membrane-protein localization (Bretscher et al., 2002). CD44, through interaction with these linker proteins, also influences these diverse cellular processes.

3.3.4 CD44 and cancer stem cells

Cancer stem cells (CSC) or cancer initiating cells (CIC) are defined as a minor population of cells within a tumor that display stem-cell properties (Zoller, 2011). They can self-renew, differentiate into different lineages and reconstitute the heterogeneous phenotype of the parental tumor they were derived from in serial xenotrasplant assays (Stamenkovic and Yu, 2009). These cells are highly chemoresistant and are thought to be essential for metastasis formation. CD44 has been identified as one of the most common markers of CSCs in many tumor entities, including leukemia, breast, colon, ovarian, prostate and pancreatic cancer (Croker and Allan, 2008).

3.3.5 CD44 in osteosarcoma

Little is known about the contribution of CD44 to OS progression and metastasis. Two immunohistochemical studies with osteosarcoma tissue specimens came to different conclusions. Kim et al. reported that overexpression of CD44v5 in tumor tissue correlated significantly with metastasis and lower survival rates (Kim et al., 2002). An analysis of tumor samples by Kuryu et al. found a correlation between the overexpression of CD44 isoforms containing variant v6 and poor prognosis (Kuryu et al., 1999). In both studies, the expression of total CD44 did not correlate with prognosis. Recent reports, also aiming at determining the

prognostic value of CD44 expression in OS, failed to find a correlation between CD44 gene or protein expression in OS tumor specimens and overall survival. However, Xu-dong et al. observed that OS patients with high expression of CD44 gene were more prone to have metastases (Boldrini et al., 2010; Xu-Dong et al., 2009). Before drawing conclusions, we have to be aware that most of the studies performed the analysis in tumor tissue of a relatively small number of patients. Thus, large cohorts of samples need to be analysed to determine the potential association of CD44 with other clinicopathological variables and its possible use as a predictor of prognosis.

Weber et al. examined in mice the effects of targeted deletion of the CD44 gene on the spontaneous development of endogenous different tumors caused by mutations in tumor suppressor genes (*min* mutation of the APC gene and *tm1* mutation of the p53 gene) (Weber et al., 2002). Mice with an APC $^{+/min}$ genotype were found to be predisposed to develop multiple benign intestinal tumors, whereas mice with trp53 $^{+/tm1}$ genotype are susceptible to a larger spectrum of tumors, predominantly sarcomas and lymphomas. When CD44-null mice were crossed with mice carrying *tm1* mutation of the p53 gene, the animals developed osteosarcomas and only one individual lung metastases was detected in 4 CD44 $^{-/-}$ mice. All 6 CD44 $^{+/+}$ mice had multiple osteosarcoma-derived metastases. The absence of the CD44 gene in trp53 $^{+/tm1}$ mice had no effect on the tumor incidence and tumor weight, as well as the life span of these animals. The phenotype of benign tumors formed in APC $^{+/min}$ mice was not altered by the absence of CD44. This study supported the idea that CD44 is crucial for metastasis promotion, but not for initiation of tumorigenesis. However, bearing in mind the low number of experimental animals analyzed in these studies, the results might have been over-interpreted.

In a differently designed study, Shiratori et al. showed that lipopolysaccharides, a proinflammatory mediator, up-regulated the expression of CD44 in murine POS-1 OS cells, which accelerated lung metastasis in a syngeneic mouse model (Shiratori et al., 2001).

Interestingly, gain or loss-of function studies investigating the role of CD44 in OS in established cell lines or xenograft mouse models have so far not been reported.

3.4 Hyaluronan

Hyaluronan (HA, hyaluronic acid) is an extracellular and cell-surface associated linear glycosaminoglycan, composed of repeating disaccharides of glucuronic acid and N-acetyl-glucosamin (Figure 8). The number of alternating units can vary depending on the tissue source and the physiological conditions, but usually HA molecules contain between 2000 and 25000 disaccharides, corresponding to molecular weights of between 10^6 and 10^7 Da and a length of 2-25 µm. In contrast to other glycosaminoglycans, HA does not include heparin sulphate and chondroitin sulphate and is not covalently bound to a core protein. It has a ubiquitous tissue distribution in vertebrates, both in the embryo and the adult, however, it is especially accumulated in pericellular matrices surrounding proliferating and migrating cells, e.g. during embryonic morphogenesis and in inflammation, wound repair and cancer (Toole, 2004).

Hyaluronic acid is produced by hyaluronan synthases (HAS1, HAS2, HAS3) that are integral plasma membrane proteins. Simultaneously with the synthesis process, HA polymers are directly extruded through the plasma membrane into the extracellular space. They can be retained at the cell surface by HA synthases or through binding to receptors (CD44, RHAMM, lymphatic vessel endothelial receptor, layilin, and Toll-like receptor-4 (Bono et al., 2001; Termeer et al., 2002; Turley et al., 2002). Hyaluronan is degraded by the hyaluronidase

family of enzymes, some of which are considered as tumor suppressors (Frost et al., 2000). HA fragmentation also occurs through reaction with reactive oxygen species (Yamazaki et al., 2003). Products generated were shown to induce angiogenesis or to provoke cleavage of CD44, thereby promoting motility and invasion (Sugahara et al., 2006; West et al., 1985).

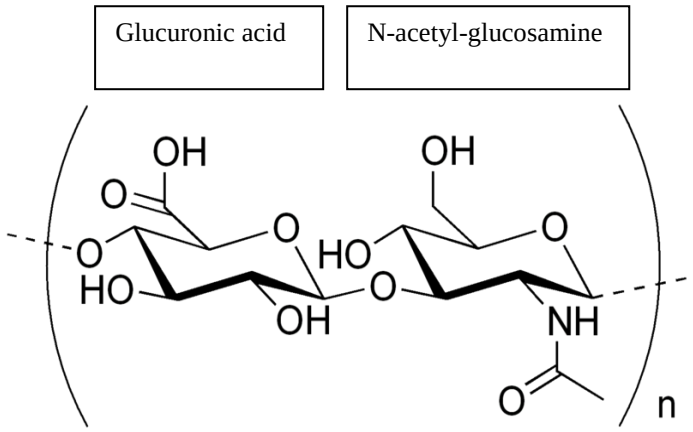


Figure 8: Chemical structure of hyaluronic acid.

Numerous biological actions of HA result from its extraordinary biophysical and interactive properties. HA contributes to tissue homeostasis and biomechanical integrity through its negative charge characteristics and its ability to retain water. Its interactions with proteoglycans and other extracellular macromolecules are crucial for the assembly and organization of extra- and pericellular matrices (Toole, 2002). Through binding to specific receptors, such as CD44 and RHAMM, HA stimulates signal transduction, either directly or by activating other receptors, and thereby influences cell behavior in various morphogenetic and physiological systems (Turley et al., 2002). For example, CD44 on cancer cells interacts with HA-rich microenvironments. This triggers cell signaling pathways that regulate migration and invasion of malignant cells through ECM and lodging at distant sites (Misra et al., 2011).

3.4.1 HA in tumor progression

Numerous studies over the last few decades revealed evidence for a tumor promoting role of HA, both in animal models and in cancer patients. It is well established that increased levels of HA, both in tumor cells and in the peritumoral stroma, are prognostic for malignant progression. In patients suffering from breast, ovarian or prostate cancers, high HA levels in tumor stroma were found associated with unfavorable outcome (Anttila et al., 2000; Auvinen et al., 2000; Lipponen et al., 2001). Increased amounts of HA have also been observed in the urine of bladder carcinoma patients (Lokeshwar et al., 2002), in the serum of breast cancer patients (Delpech et al., 1990) and in the saliva of patients with head and neck cancer (Franzmann et al., 2003). However, no correlation was found between the HA levels in melanoma tissue and tumor progression (Karjalainen et al., 2000).

The results of studies in animal models investigating the role(s) of HA in tumor progression are contradictory. Several approaches have been used to prove the contribution of HA to tumor progression, such as manipulation of HA levels and perturbation of endogenous HA-protein interactions in various ways. Overexpression of HAS1, HAS2 or HAS3 followed by overproduction of HA in tumor cells resulted in increased growth or metastatic activity of tumors in xenograft models of fibrosarcoma (Kosaki et al., 1999) and of prostate (Liu et al., 2001), breast (Itano et al., 1999) and colon cancer (Jacobson et al., 2002). Additional experimental proof for a tumor-promoting role of HA was obtained in studies, which demonstrated that overexpression of hyaluronidases suppressed the growth of colon and breast carcinoma xenografts (Jacobson et al., 2002; Shuster et al., 2002). However, some reports claim the contrary and provide evidence for a tumor-promoting effect of hyaluronidase overexpression in astrocytoma and prostate cancer cell lines (Novak et al., 1999; Patel et al., 2002). These findings obtained in experimental tumor models are consistent with clinical data indicating increased levels of hyaluronidase (usually HYAL1) in bladder

(Hautmann et al., 2001), prostate (Posey et al., 2003) , head and neck (Franzmann et al., 2003) and brain cancer (Delpech et al., 2002).

All findings taken together emphasize an important regulatory role of HA in cancer progression.

3.4.2 HA in osteosarcoma

Several groups investigated the impact of HA on osteosarcoma progression. The study of Nishida et al. (Nishida et al., 2005) revealed that inhibition of HAS2 expression in human MG63 OS cells by antisense phosphorothioate oligonucleotides reduced HA production and resulted in the disruption of cell-associated matrices assembly and, consequently, in decreased cell proliferation, motility and invasive capacities. Tofuku et al. found that HA synthesized by HAS3 promoted biological functions crucial for metastasis, such as proliferation, invasion and degradation of extracellular matrix. 4-methylumbelliferon (4-MU), an inhibitor of HA synthesis, was shown to inhibit both proliferation and invasion of LM8 cells in vitro (Tofuku et al., 2006). Hosono et al. (Hosono et al., 2007) examined the effects of HA oligosaccharides on tumorigenicity of LM8 murine OS cells and MG63 human osteosarcoma cells. They reported that treatment with HA octamers suppressed the formation of cell-associated matrix, which resulted in the inhibition of growth, motility and invasiveness and the induction of apoptosis in vitro in both cells lines. In in vivo studies with LM8 cells subcutaneously injected into syngeneic mice, intratumoral injection of HA oligosaccharides reduced the accumulation of HA in tumor tissue and resulted in significant suppression of lung metastasis. In a recent study, the same group showed that 4-MU effectively inhibits various processes of tumorigenicity in vitro in murine LM8 and human MG63 and HOS OS cells. Administration of 4-MU in vivo markedly suppressed lung metastasis of the highly metastatic LM8 OS cells (Arai et al., 2011).

Taken together, these reports highlight the involvement of HA in the progression and metastasis of OS.

3.5 Aim of the Thesis

CD44 is a multifunctional protein that has been implicated in different aspects of tumor progression, especially with the metastatic spread of different types of cancer as described above. CD44 is the principle receptor for HA, and their interaction promotes the malignant behavior of various tumor cell types, whereas it is not essential for the metastatic behavior of other tumor cell types. The overall aim of this thesis was to investigate the contribution of CD44 and HA interaction to OS primary tumor development and metastasis. In order to accomplish our goal to gain better understanding on the role of CD44 and HA interaction in OS biology we had to complete two specific objectives.

The first objective was to estimate the prognostic value of CD44 expression for OS patients' outcome.

The second objective was to explore the biological relevance of CD44 and HA interaction for *in vitro* malignant properties of OS tumor cells and for *in vivo* OS progression and metastasis using several orthotopic xenograft OS mouse models.

4 Results

4.1 Manuscript 1:

CD44 Enhances Tumor Formation and Lung Metastasis in Experimental Osteosarcoma and is an Additional Predictor for Poor Patient's Outcome

Ana Gvozdenovic,[1] Matthias JE Arlt,[1] Carmen Campanile,[1] Patrick Brennecke,[1] Knut Husmann, [1] Yufei Li[1,2], Walter Born,[1] Roman Muff,[1] and Bruno Fuchs[1]

[1]Laboratory for Orthopedic Research, Department of Orthopedics, Balgrist University Hospital, Zurich, Switzerland

[2]Institute for Biomechanics, Swiss Federal Institute of Technology (ETH), Zurich, Switzerland

Address correspondence to: Bruno Fuchs, MD, PhD, Laboratory of Orthopedic Research, Balgrist University Hospital, Forchstrasse 340, CH-8008 Zurich, Switzerland. E-mail: bfuchs@research.balgrist.ch

Additional Supporting Information may be found in the online version of this article.

Disclosures

All the authors state that they have no conflicts of interest.

ABSTRACT

Formation of metastases in the lungs is the major cause of death in patients suffering from osteosarcoma (OS), a disease mainly affecting children and adolescents. Metastases at presentation and poor response to preoperative chemotherapy are strong predictors for poor patient's outcome. The elucidation of molecular markers that promote metastasis formation and/or chemoresistance is therefore of importance. CD44 is a plasma membrane glycoprotein that binds to the extracellular matrix component hyaluronan (HA) and has been shown to be involved in metastasis formation in a variety of other tumors. Here we investigated the role of CD44 expression on OS tumor formation and metastasis. High CD44 expression, evaluated with a tissue microarray including samples from 53 OS patients and stained with a pan CD44 antibody (Hermes3), showed a tendency ($p < 0.08$) to shortened overall survival. However, non-responders and patients with lung metastases and high CD44 expression had significantly poorer prognosis than patients with low CD44 expression. Overexpression of CD44 (standard isoform CD44s) and its hyaluronan binding defective mutant R41A in osteoblastic SaOS-2 cells resulted in HA-independent higher migration rates and increased chemoresistance, partially dependent on HA. In an orthotopic mouse model of OS, overexpression of CD44s in SaOS-2 cells resulted in a HA-dependent increased primary tumor formation and increased numbers of micro- and macrometastases in the lungs. In conclusion, although CD44 failed to be an independent predictor for patient's outcome in this limited cohort of OS patients, increased CD44 expression was associated with even worse survival in patients with chemoresistance and with lung metastases. CD44 associated chemoresistance was also observed *in vitro*, and increased formation of lung metastases was found *in vivo* in SCID mice.

KEY WORDS: CD44; CHEMORESISTANCE; HYALURONAN; METASTASIS; OSTEOSARCOMA

Introduction

Osteosarcoma (OS) is the most common primary tumor of bone in children and adolescents. The presence of malignant spindle cells that produce osteoid and/or immature bone is characteristic for this highly aggressive cancer type.[1] The incidence of OS in the general population is 3 cases per million per year, but is higher in adolescence, in which it reaches 8-11 cases/million/year at 15-19 years of age.[2] OS has a great tendency of spreading to the lungs, and less frequently to the bones. Formation of bone metastases occurs only after pulmonary metastases have already been established.[3] At the time of diagnosis, up to 15-20% of patients already have detectable metastases. However, 80% of patients initially presenting with localized disease develop metastases after surgical resection.[4] Combination of multi-agent chemotherapy with surgery introduced in late 1970's remarkably improved the overall survival of patients with non-metastatic disease, whose 5-year survival rate is now 70%, as opposed to only 20% few decades ago. In contrast, the patients with metastatic or recurrent disease did not benefit from these clinical advances and they unfortunately face a very poor prognosis, with a survival rate that remains still at 20%.[5] The failure of treatment in these patients is often associated with gained resistance to chemotherapy.[6] Nowadays, the most powerful and reproducible prognostic indicators for OS patients are metastatic lesions at presentation and histological response to preoperative chemotherapy.[7] Thus, it is of substantial relevance to identify molecular markers associated with the increased metastatic potential or chemoresistance, which may serve as diagnostic or prognostic factors. Acquiring insight into the basic biology of OS progression will make the identification of such new therapeutic targets possible with the final goal to develop treatment strategies that eradicate metastases, the major cause of death in OS.

CD44 has been linked with increased metastatic spread in various types of cancer.[8] CD44 designates a family of broadly distributed type I transmembrane glycoproteins that serve as

cell-cell and cell-matrix adhesion molecules and as principal receptors for hyaluronan (HA), a major component of the extracellular matrix in many tissues including bone.[9] Existence of multiple isoforms, generated through alternative splicing, and extensive post-translational modifications underlie the wide repertoire of CD44 biological functions in development, wound healing, inflammation, hematopoiesis, immune response and tumor progression.[10] Tissue-specific splicing results in the formation of the standard CD44 isoform (CD44s), lacking all variant exons, in cells of mesenchymal origin and, thus, the expression of this isoform may be relevant for sarcoma tumor progression.[11,12] CD44 has been shown to promote tumor and metastasis development both in a HA-dependent and HA-independent fashion.[13,14] Both CD44 and HA have been implicated in resistance to anticancer drugs.[15-17]

Only few immunohistochemical studies using OS tissue specimens addressed the contribution of CD44 to OS progression and metastasis giving rise to conflicting data.[18-20] Interestingly, gain or loss-of function studies investigating the role of CD44 in established OS cell lines or xenograft mouse models, to our knowledge, have so far not been reported. On the other hand, a limited number of reports indicated the relevance of HA in OS tumor progression after making use of established cell lines. Treatment with HA oligosaccharides suppressed the formation of cell-associated matrix, leading to inhibited tumorigenicity of the human MG63 and murine LM8 OS cell lines *in vitro*.[21] In *in vivo* studies, intratumoral injection of HA oligosaccharides into subcutaneous LM8-derived tumors reduced the accumulation of HA in tumor tissue and resulted in significant suppression of lung metastases.[21] *In vivo* administration of 4-methylumbelliferon, an inhibitor of HA synthesis, inhibited the retention of HA in the periphery of the primary tumors and markedly reduced the number of metastatic lung lesions formed by LM8 OS cells.[22]

In our current study we show that CD44 can be used as an additional prognostic factor for OS patients' outcome. With the aim to investigate the biological relevance of CD44s expression

and its' interaction with HA for OS progression and metastasis, we overexpressed the CD44s isoform and its' HA-binding defective mutant CD44s R41A in the low metastatic human OS SaOS-2 cells, that display an osteoblastic phenotype most commonly observed in human patients. Using an intratibial xenograft OS mouse model we demonstrated that CD44 standard isoform enhances primary tumor growth and formation of pulmonary metastases in a HA-dependent manner. In conclusion, the results presented here highlight CD44-HA interaction as a potential target for therapeutic intervention in OS.

Materials and Methods

Human OS tissue microarray analysis

OS tissue specimens were collected between June 1990 and December 2005 from 53 patients during primary tumor resection in accordance with the regulations of the local ethic committee. Clinical data of the patients are presented in Table 1. All patients received neoadjuvant chemotherapy and the subsequent response was determined histologically on resected tumor specimens according to Salzer-Kuntschik.[23] Grades I, II and III were considered as a good response, whereas grades IV, V and VI were classified as a poor response. The tissue microarray was arranged as described.[24] Microarray sections of 4.5 µm were processed as reported [25] and stained with a pan-CD44 antibody Hermes3 (generously provided by Dr. S. Jalkanen, Turku, Finland) (1:1000) and counterstained with hematoxylin. Tissue microarray grading was performed based on the intensity and area percentage of the positive stain using Table 1 in Supplemental Data. The intensity of the stain was judged by eye (week, moderate and strong). The percentage of staining was calculated using a custom MATLAB (v2009b, Mathworks, MA) program. Positive (brown) and negative (blue) staining were separated using color deconvolution theory.[26] The area percentage of the stain was defined as positive stained area (number of brown pixels) over total tissue area (number of

blue and brown pixels) (Fig. 1, Supplemental Data). Kaplan-Meier analysis was used to correlate CD44 expression with overall and event-free survival of OS patients.

Cell culture and transduction

Human OS SaOS-2 (HTB-85) cells obtained from American Type Culture Collection (ATCC, Rockville, MD, USA), were cultured in DMEM (4.5 g/l glucose)/HamF12 (1:1) medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat inactivated FCS at 37℃ in a humidified atmosphere of 5% CO_2/95% air. In order to enable visualization of tumor cells within mouse tissues, SaOS-2 cells were transduced with *LacZ* gene (SaOS-2/*LacZ* cells) as described recently.[27] Stable expression of the standard CD44 isoform (CD44s) and its' HA binding-defective mutant CD44s R41A was achieved by retroviral gene transfer. Briefly, pMSCV vectors containing human CD44s and CD44s R41A coding sequences, generously provided by Prof. Stamenkovic (Lausanne, Switzerland), were used to fuse V5 and His6 epitopes to the COOH-terminal ends of CD44s and CD44s R41A giving rise to CD44s-V5/His6 and CD44s R41A-V5/His6 encoding sequences that were subsequently subcloned into the retroviral expression vector pQCIXH (Clontech, Paolo Alto, CA, USA) containing a hygromycin resistance gene. All expression constructs were verified by sequencing of both strands. Retroviral particles containing pQCIXH EV (empty vector), pQCIXH CD44s-V5/His6 and pQCIXH CD44s R41A-V5/His6 were produced in HEK 293T cells, and were subsequently used to infect SaOS-2/*LacZ* cells as described.[27] Selection for hygromycin resistance in medium containing 400 µg/ml of hygromycin (Calbiochem, Switzerland) revealed SaOS-2 EV, SaOS-2 CD44s and SaOS-2 CD44s R41A cell lines.

Western blot analysis

Cells were lysed by agitation on a carrousel at 4℃ for 1h in lysis buffer containing 50 mM Tris/HCl (pH 7.5), 150mM NaCl, 1% NP40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF)

and 10 mg/ml aprotinin. Cellular debris were removed by centrifugation at 13 000 rpm and 4℃ for 20 minutes. Equal amounts of proteins of individual cell extracts were separated by 8% SDS-PAGE. The proteins were then transferred by semi-dry blotting to Hybond-ECL membranes (GE Healthcare, UK). Endogenous and V5-tagged CD44 proteins and GAPDH were visualized with respective mouse monoclonal Hermes3 antibody (concentration 1µg/ml), V5 antibody (1:5000; Invitrogen) and rabbit polyclonal anti-GAPDH antibody (1:3000; Santa Cruz Biotechnologies, CA, USA) and corresponding HRP-conjugated secondary antibodies purchased from Santa Cruz Biotechnologies. Peroxidase activity was detected with the Immobilon chemoluminescence substrate (Millipore, Billerica, MA, USA) and a VersaDoc^{TM} Imaging System (Bio-Rad, Hercules, CA, USA).

Adhesion assay

96-well plates were coated with 333 µg/cm^2 of high molecular weight HA (HMW-HA) (Sigma-Aldrich, St. Luis, MO, USA) at 4℃ over night. They were then washed with PBS and blocked with heat-denatured 1% BSA (HD-BSA). Non-coated wells or wells coated with HD-BSA alone were used as controls. Adhesion assays were carried out with cells grown in tissue culture medium to subconfluency in 25cm^2 tissue culture flasks. They were then detached with accutase (Sigma-Aldrich), resuspended in medium and seeded at 10^4 cells per well and allowed to adhere at 37℃ for 30 minutes. In CD44 blocking experiments, adhesion was performed in the presence of 10 µg/ml Hermes1 antibody (kindly provided by Dr. S. Jalkanen, Turku, Finland) that blocks HA binding or of 10 µg/ml rat IgG_{2A} antibody (R&D Systems, Minneapolis, MN, USA) as a control. Non-adherent cells were removed by washing with PBS and adherent cells were fixed with 10% formalin in PBS at room temperature (RT) for 15 min and then stained with 0.05% crystal violet in H_2O at RT for 15 min. Images of randomly selected areas of 3.6 mm^2 were taken with an AxioCam MRm camera connected to the Zeiss Observer.Z1 inverted microscope (Carl Zeiss MicroImaging GmbH, Göttingen,

Germany) set at at 4x magnification. The number of adherent cells in the analyzed area was estimated with ImageJ software. The total number of adherent cells per well was calculated and the percentage of adherent cells was obtained by dividing the number of adherent cells by the total number of seeded cells and multiplying with 100. The experiments were performed in triplicates and repeated three times.

Transwell migration assay

Cell culture inserts (Becton Dickinson, San Jose, CA, USA) with 8 µm porous filters in 24-well plates were used for a transwell migration assay. Cells grown to subconfulency were detached with accutase (Sigma-Aldrich) and 2 x 10^4 cells in 300 µl of serum-free cell culture medium supplemented with penicillin/streptomycin/amphotericin B (PSA, 1:100; Invitrogen) were added to the upper compartment of the inserts. The lower compartments were filled with 700 µl of medium containing 10% FCS complemented with PSA. After incubation at 37℃ for 24h, non-migrating cells on the upper side of the insert were removed with a cotton swab. Cells that had migrated to the lower side of the filters were fixed with 10% formalin, permeabilized with 50 µM digitonin (Calbiochem, Switzerland) and stained with 300 nM Picogreen in PBS (1:200; Invitrogen) at RT for 15 min. Three images per insert (two inserts per cell line) showing an area of 0.58 mm^2 were taken with an AxioCam MRm camera connected to the Zeiss Observer.Z1 inverted microscope adjusted to 10x magnification and equipped with an appropriate filter block for blue excitation. The number of cells on the images was counted with the ImageJ software, and the percentage of migrated cells was calculated as described for the adhesion assay. The experiments were performed at least three times.

In vitro cell proliferation assay

Subconfluent cells in the logarithmic growth phase were trypsinized and 5 x 10^4 cells, resuspended in 2.5 ml of cell culture medium, were seeded in 12.5 cm^2.The cells were allowed to grow for between 1 and 7 days and counted in intervals of 48 hours in triplicates. Cells in individual flasks were detached by trypsinization and counted in a Neubauer chamber. The doubling time during logarithmic growth was calculated according to the equation $N=N_o\ e^{kt}$ (N_0= number of seeded cells; N=number of cells at time t). The experiments with individual cell lines were carried out three times.

Cytotoxicity assay

3 x 10^3 cells per well were seeded in 96-well plates and allowed to adhere overnight. The cells were then incubated in duplicates with increasing concentrations of cisplatin (0.01 - 25 µg/ml), doxorubicin (0.01 – 6 µg/ml) and etoposide (0.05 – 250 µg/ml) for 72h. All drugs were purchased from Sigma-Aldrich. After the drug treatment, the cells were incubated with 10 µl/well of WST-1 reagent (Roche, Switzerland) for 3 hours and the cell viability was then assessed as reported.[28] Prism 4 network software was used to calculate the half-maximal growth inhibitory concentration (IC_{50}) of the drugs. The experiments were repeated three times.

Intratibial OS xenograft model in SCID mice

The animal experiments were performed as described [25] according to the guidelines of the "Schweizer Bundesamt für Veterinärwesen" and as approved by the authorities of the Kanton Zürich. Briefly, on day 0 of the experiment, 5 x 10^5 of SaOS-2 cells engineered as indicated were suspended in 10 µl of PBS/0.05% EDTA and injected intratibially into SCID/CB17 immunocompromised mice obtained from Charles River Laboratories (Sulzfeld, Germany). After the injection, the health condition of the mice was closely monitored. The development

of primary tumors was visualized bi-weekly during the first 1 to 9 weeks of the experiment and then weekly until the end of the experiment by X-ray with a MX-20 DC Digital Radiography System (Faxitron X-Ray Corporation Lincolnshire, IL, USA). The tumor volume was further estimated by measuring the length and the width of the tumor leg with a caliper, and the volume was calculated according to the formula V= length x width^2/2. The volume of the non-injected leg was used as a reference value. The mice were sacrificed in week 12 after tumor cell injection and the lung was perfused in situ as described.[25] Primary tumors and lungs were fixed at RT in 2% formaldehyde for 30 minutes and processed for X-gal staining as reported.[27] Indigo-blue stained metastases on the surface of lung whole mounts were counted at 4x magnification under the Nicon Eclipse E600 microscope (Nikon Corporation, Tokio, Japan) equipped with an integrated size grid. Metastatic foci smaller than 0.1 mm in diameter were considered as micrometastases and foci bigger than 0.1 mm as macrometastases. Two independent animal experiments were performed and the data were pooled.

Statistical analysis

Overall and event-free patient survival was calculated using Kaplan–Meier curves and statistical significance was assessed with the log-rank test. Differences between means were analyzed by the Student *t*-test and $p < 0.05$ was considered significant. The results are presented as means ± standard errors of the mean (SEM).

Results

CD44 expression in human OS tumor samples is an additional prognostic factor in non-responders and in patients with lung metastases

Human OS tissue-microarray sections including tumor specimens from 53 patients were analyzed immunohistochemically for total CD44 expression (Fig. 1). The adequacy of our

patient cohort was evaluated by determining the correlation of chemotherapy response and the presence of metastases with the overall survival, as these were identified as key determinants of prognosis in OS.[29] Indeed, non-responders and metastases-positive patients had significantly shorter overall survival ($p < 0.05$, $p < 0.0001$, respectively) than responders and metastases-free patients (not shown). Patients poorly responding to chemotherapy developed lung metastases more rapidly, and had a mean event-free survival of 14 ± 2 months compared to 40 ± 2 months in patients with good response ($p < 0.05$; Fig. 1*B*). A Kaplan-Meier analysis revealed a tendency of shorter overall mean survival (50 ± 8 months) of patients with positive CD44 staining in tumor resections than patients with undetectable CD44 staining (88 ± 8 months; $p = 0.0758$; Fig. 1*C*). Non-responders that were CD44 positive had a tendency of shorter overall survival than CD44 negative patients ($p = 0.0732$; Fig. 1*D*). In addition, non-responders with CD44 positive staining in their tumor samples had significantly shorter mean event-free survival of only 8.3 ± 1.4 months than patients with undetectable CD44 in tumor sections with mean event-free survival of 16.5 ± 3 months ($p < 0.05$; Fig. 1*E*). Strikingly, all patients that were positive for both CD44 expression and metastases died within 22 months significantly earlier than CD44 negative patients ($p < 0.0001$; Fig. 1*F*). The findings implicate that CD44 is an additional negative predictor for OS patients' outcome, in addition to the commonly used prognostic parameters such as chemotherapy response and presence of metastases.

Overexpression of CD44 in an osteoblastic OS cell line increases the adhesion to HA, promotes cell migration and induces chemoresistance

Based on the tissue microarray results, we hypothesized that CD44 may have a significant impact on the metastatic activity and the chemoresistance of OS cells. We therefore overexpressed by retroviral gene transfer the C-terminally V5/His6-tagged standard isoform CD44s in the human low metastatic SaOS-2 OS cell line with low endogenous CD44

expression. The standard isoform, with all the variant exons excised, was chosen for overexpression because it was found expressed as the predominant isoform in other human OS cell lines (not shown). The V5/His6-tagged HA binding-defective mutant CD44s R41A was included in the study to assess the relevance of CD44/HA interactions in the regulation of the metastatic ability and chemoresistance of SaOS-2 tumor cells. Western blot analysis of whole cell extracts with a pan-CD44 antibody (Hermes3) indicated overexpression of CD44s and CD44s R41A in respective SaOS-2 CD44s and SaOS-2 CD44s R41A cells compared to control SaOS-2 EV (Fig. 2*A*). The protein components detected by Hermes3 and V5 antibodies had the expected size of approximately 100 kDa.

A significant ($p < 0.01$) 4.2-fold higher percentage of HA-adhering SaOS-2 CD44s cells compared to SaOS-2 EV cells in an assay examining short-term (30 minutes) adhesion demonstrated the functional expression of CD44s at the cell surface (Fig. 2*B*). Consistent with the binding defect of CD44s R41A, the adhesion of SaOS-2 CD44s R41A cells to HA was indistinguishable from that of SaOS-2 EV cells. A significant reduction in the percentage of short-term adhering SaOS-2 CD44s cells by preincubation with Hermes1 CD44s blocking antibodies, which was not observed with control IgG, further confirmed that increased adhesion of SaOS-2 CD44s cells to HA was indeed mediated by direct interaction of overexpressed CD44s at the cell surface with HA (Fig. 2*C*). Interestingly, pretreatment of SaOS-2 CD44s cells with Hermes1 antibodies inhibited their adhesion to HA to a percentage comparable to that of control SaOS-2 EV cells, indicating that CD44s blocking was almost complete. It is also important to note that pretreatment of SaOS-2 EV and of SaOS-2 CD44s R41A cells with Hermes1 did not affect short-term adhesion of the two cell lines to HA (not shown).

Tumor cell migration, another indicator of metastatic potential *in vitro*, was investigated with the CD44s expression-manipulated cells in a transwell migration assay. The migration rates of

SaOS-2 CD44s and SaOS-2 CD44s R41A cells were 4-fold ($p < 0.05$) and 3-fold ($p < 0.05$) higher than that of SaOS-2 EV cells (Fig. 2*D*) suggesting that the CD44s expression-related increase in migration activity was not dependent on CD44s/HA interactions. On the other hand, effects of CD44s overexpression on the proliferation of SaOS-2 cells was not observed. The calculated doubling times were 38.2 ± 1.6h for SaOS-2 EV cells, 42.1 ± 4.7h for SaOS-2 CD44s and 46.7 ± 5.2h for SaOS-2 CD44s R41A cells (Fig. 2*E*). Thus, overexpression of CD44s in SaOS-2 cells enhanced the *in vitro* metastatic properties such as adhesion and migration in HA-dependent and HA-independent manner, respectively, without affecting cell proliferation.

The tissue microarray analysis of OS resections also suggested that expression of CD44 in primary tumor tissue is related to and may even directly enhance the resistance to commonly used chemotherapeutics in OS patients. The here presented results of cytotoxicity experiments with CD44s and CD44s R41A overexpressing and control SaOS-2 EV cells supported this hypothesis. The half-maximal growth inhibitory concentration (IC_{50}) of cisplatin was 2.4-fold higher ($p < 0.01$) in SaOS-2 CD44s cells than in control SaOS-2 EV cells (Fig. 2*F*). This significant increase in chemoresistance was not observed with the HA binding-defective CD44s R41A mutant implying that increased cisplatin resistance was only partially HA-dependent. A tendency of increased chemoresistance of SaOS-2 CD44s cells to doxorubicin and etoposide was also found (not shown).

CD44s enhances intratibial primary tumor growth and pulmonary metastasis in a xenograft OS mouse model in a HA-dependent manner

The *in vitro* experiments showed that CD44s, when overexpressed in SaOS-2 cells, enhances adhesion and migration, cellular processes essential for the metastatic progression. We therefore compared the growth and metastasis of SaOS-2 EV, SaOS-2 CD44s, SaOS-2 CD44s

R41A cell line-derived intratibial primary tumors in SCID mice. Osteoblastic lesions were first observed by X-Ray in mice that were injected with SaOS-2 CD44s cells 50 days after tumor cell injection (Fig. 3*A*). SaOS-2 EV and CD44s R41A cell-derived tumors developed more slowly and the first osteoblastic lesions became visible 2 weeks after those in mice injected with SaOS-2 CD44s cells. At the end of the experiment on day 90 after tumor cell injection, SaOS-2 CD44s cell-derived tumors showed more extensive bone structures on X-ray images than tumors of SaOS-2 EV and SaOS-2 CD44s R41A cells. The animals became moribund in the 12^{th} week after tumor cell injection and were subsequently sacrificed. Finally, CD44s-xenografts with a mean primary tumor volume of 226 ± 29 mm^3 were significantly ($p < 0.05$) larger than SaOS-2 EV (121 ± 25 mm^3) and SaOS-2 CD44s R41A (130 ± 31 mm^3) xenografts (Fig. 3*B*). Moreover, the ex-vivo analysis of whole mounts of lungs revealed 2.5 and 2.2 times higher numbers of X-gal-stained macrometastases in SaOS-2 CD44s than in SaOS-2 EV and SaOS-2 CD44s R41A tumor bearing mice, respectively ($p < 0.01$; Fig. 4*B*). Similarly, the mean number of micrometastases was 2- and 2.2 times higher on the lungs of mice with SaOS-2 CD44s cell-derived tumors than on the respective lungs dissected from SaOS-2 EV and SaOS-2 CD44s R41A tumor bearing animals ($p < 0.05$; Fig. 4*C*). The significant differences in tumor size and in the mean number of lung macro- and micrometastases in SaOS-2 CD44s compared to SaOS-2 CD44s R41A tumor bearing mice clearly indicated that the observed CD44s-promoted enhanced malignancy of SaOS-2 CD44s cells was dependent on interaction with HA.

Discussion

Osteosarcoma is the second leading cause of cancer-related death in pediatric age group and young adults.[30] This high mortality is due to development of pulmonary metastases that are detectable already in 15-20% of the patients at the time of diagnosis. In order to devise successful disease management strategies and to improve the survival rate of OS patients, it is

essential to gain a profound understanding of OS and metastasis progression as well as associated chemoresistance mechanisms. Identification of novel OS molecular markers that will serve as therapeutic targets is crucial for the design of effective treatment of this devastating disease.

In the present study, we investigated the prognostic value of CD44 expression for OS patients' outcome, as well as the biological relevance of CD44-HA interactions in an orthotopic xenograft OS mouse model. In our tissue microarray analysis, we evaluated the predictive value of total CD44 protein expression. CD44s is probably the main isoform expressed in OS samples, as it is the predominant isoform expressed in several established OS cells lines (not shown) that are of mesenchymal origin. This is in line with a study conducted by Brown et al. where the authors demonstrate that a switch in expression from CD44 variant isoforms to CD44s is essential for epithelial-mesenchymal transition and that mesenchymal CD44s is upregulated in advanced human breast carcinomas.[31] On the other hand, our suggestion is in contrast with two studies that found variant CD44 isoforms to be correlated with poor survival of OS patients.[18,19] We did not find a significant correlation between CD44 expression and overall survival, which is in good agreement with recent reports of Boldrini et al. and Xu-Dong et al.[20,32] Interestingly, Xu-Dong et al. observed that OS patients with high expression of CD44 gene were more prone to have metastases.[32] Our Kaplan-Meier analysis, however, revealed a significant correlation between CD44 expression in OS surgical tumor specimens of patients with a poor response to preoperative chemotherapy and shorter event-free survival. Moreover, CD44 positive patients with metastasis had a significantly shorter overall survival when compared to CD44 negative patients with metastatic disease. Despite the limited number of patient samples analyzed due to the low incidence of OS, our data indicate that CD44 expression is an additional prognostic indicator together with chemotherapy response and metastasis presence that are commonly employed in

the clinics as parameters for predicting clinical outcomes among OS patients.[7] In the emerging era of personalized oncology care, the ultimate goal is to tailor therapy to individual patients according to distinct characteristics of their tumors in order to enhance clinical efficiency while minimizing the adverse toxic side effects of antineoplastic drugs.[33] We suggest CD44 as a molecular marker that can be used to identify subgroups of patients with different prognosis and that can further be a basis for individualized treatment. Stratification of patients into different-risk categories may guide the choice of postoperative chemotherapy. Our findings identify CD44 positive patients within non-responders and patients with metastatic disease as high-risk patients that, therefore, may be considered as candidates for intensified chemotherapy or novel therapeutic strategies.

Based on our tissue microarray analysis, we speculated that CD44 may be an important player in the metastatic behavior and chemoresistance of OS cells. In fact, making use of a xenograft orthotopic mouse model of OS, we demonstrated that CD44s has a tumor- and metastatic-promoting activity upon overexpression in a human osteoblastic SaOS-2 cell line. The malignancy enhancing effect of CD44s was HA-dependent and was reflected in the significant increase of primary tumor volume and the numbers of pulmonary micro- and macrometastases when compared to that of SaOS-2 EV and SaOS-2 CD44s R41A cells. Apparently, CD44s affected both dissemination of tumor cells and their growth re-establishment at the secondary site. *In vitro* experiments showed that overexpression of CD44s promotes adhesion to HA and facilitated migration of SaOS-2 cells. The observed increase in migration rates upon overexpression of CD44s did not rely on the binding capacity to HA. This observation is not surprising given the fact that CD44 also functions as a co-receptor of several receptor tyrosine kinases, such as c-Met and members of ERBB receptor family, and can thereby modulate the signaling of associated growth factor receptors.[10]

We did not observe any effects of CD44 overexpression on proliferation *in vitro* as opposed to the *in vivo* experiments where CD44s overexpressing cells formed bigger tumors in comparison to EV and CD44s R41A cells upon intratibial injection. These findings once again show that *in vitro* conditions do not fully replicate the physiological situation and highlight the major impact of tumor microenvironment on tumor development, which is already well established. Furthermore, additionally to promoting migration, CD44s induced *in vitro* chemoresistance of SaOS-2 cells to cisplatin which is in good agreement with our tissue microarray results. The CD44s effect on response to this cytotoxic drug appeared to be only partially dependent on HA binding ability which implicates involvement of additional mechanisms. In the past years, several lines of evidence indicated that HA and CD44 promote chemoresistance in a wide spectrum of tumor cell types, including breast, lung, head and neck carcinomas and lymphoma.[34] It was shown that CD44/HA interaction regulates drug transporter expression in breast carcinoma cells.[17] CD44 is associated with multidrug transporters in malignant peripheral nerve sheath tumor cells, and disruption of CD44/HA interactions abrogated drug resistance in these cells.[35] Recently, Xu et al. reported evidence that CD44 attenuates the activation of the Hippo signaling pathway and consequently promotes resistance of glioma cells to cytotoxic-agent induced stress.[16] At this point, it is important to notice that in numerous types of human carcinomas CD44 is a common marker for cancer stem cells (CSC), that are highly resistant to chemotherapy.[15] The underlying mechanism of CD44 induced chemoresistance in OS still remains to be elucidated. The future research should be directed towards confirming CD44 induced chemoresistance in animal models of OS.

In summary, our study identified CD44 as an additional negative predictor for OS patients' outcome that enables stratification of patients into subgroups which may lead to more efficient personalized therapy. With the SaOS-2 model we developed a system that allows

investigating the relevance of individual CD44 isoforms for the tumor biology of osteoblastic OS in a cellular background of insignificant endogenous CD44 expression. Findings presented here underscore the important role of CD44s/HA interaction in determining tumor malignancy in experimental OS. Taken together, our results point to CD44/HA interaction as a promising target for therapeutic intervention in OS.

Acknowledgments

We thank PD. Dr. Beata Bode (University Hospital Zurich) for the support in generating and staining of the tissue microarray and Prof. Ivan Stamenkovic (University of Lausanne) for helpful discussion. We appreciate the excellent technical assistance provided by Josefine Bertz and Christopher Bühler.

This study was supported in part by the Zurcher Krebsliga (Zurich, Switzerland), the Swiss National Science Foundation (SNF Grant Nr.31003A-120403), the University of Zurich, the Schweizerischer Verein Balgrist (Zurich, Switzerland), the Walter L. & Johanna Wolf Foundation (Zurich, Switzerland), and the Lydia Hochstrasser Stiftung (Zurich, Switzerland).

Author's roles: Study design: AG, RM, BF, WB; Experiment conduct: AG; Animal work: AG, MJEA, CC, PB; Contributed material: KH; Contributed analysis tools: YL; Data analysis: AG; Manuscript preparation: AG, RM, WB, BF.

References

1. Picci P. Osteosarcoma (osteogenic sarcoma). Orphanet J Rare Dis. 2007; 2:6.
2. Ritter J, Bielack SS. Osteosarcoma. Ann Oncol. 2010; 21 Suppl 7:vii320-5.
3. Ta H, Dass C, Choong P, et al. Osteosarcoma treatment: state of the art. Cancer and Metastasis Reviews. 2009; 28:247-63.
4. Marina N, Gebhardt M, Teot L, et al. Biology and Therapeutic Advances for Pediatric Osteosarcoma. Oncologist. 2004; 9:422-41.
5. Longhi A, Errani C, De Paolis M, et al. Primary bone osteosarcoma in the pediatric age: state of the art. Cancer Treat Rev. 2006; 32:423-36.
6. Gorlick R, Khanna C. Osteosarcoma. J Bone Miner Res. 2010; 25:683-91.
7. Meyers PA, Gorlick R. Osteosarcoma. Pediatr Clin North Am. 1997; 44:973-89.
8. Naor D, Nedvetzki S, Golan I, et al. CD44 in cancer. Crit Rev Clin Lab Sci. 2002; 39:527-79.
9. Aruffo A, Stamenkovic I, Melnick M, et al. CD44 is the principal cell surface receptor for hyaluronate. Cell. 1990; 61:1303-13.
10. Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. Nat Rev Mol Cell Biol. 2003; 4:33-45.
11. Marhaba R, Zoller M. CD44 in cancer progression: adhesion, migration and growth regulation. J Mol Histol. 2004; 35:211-31.
12. Bacac M, Stamenkovic I. Metastatic cancer cell. Annu Rev Pathol. 2008; 3:221-47.
13. Misra S, Heldin P, Hascall VC, et al. Hyaluronan-CD44 interactions as potential targets for cancer therapy. FEBS J. 2011; 278:1429-43.
14. Orian-Rousseau V. CD44, a therapeutic target for metastasising tumours. Eur J Cancer. 2010; 46:1271-7.
15. Zoller M. CD44: can a cancer-initiating cell profit from an abundantly expressed molecule? Nat Rev Cancer. 2011; 11:254-67.
16. Xu Y, Stamenkovic I, Yu Q. CD44 attenuates activation of the hippo signaling pathway and is a prime therapeutic target for glioblastoma. Cancer Res. 2010; 70:2455-64.
17. Misra S, Ghatak S, Toole BP. Regulation of MDR1 expression and drug resistance by a positive feedback loop involving hyaluronan, phosphoinositide 3-kinase, and ErbB2. J Biol Chem. 2005; 280:20310-5.
18. Kim HS, Park YB, Oh JH, et al. Expression of CD44 isoforms correlates with the metastatic potential of osteosarcoma. Clin Orthop Relat Res. 2002:184-90.
19. Kuryu M, Ozaki T, Nishida K, et al. Expression of CD44 variants in osteosarcoma. J Cancer Res Clin Oncol. 1999; 125:646-52.
20. Boldrini E, Peres SV, Morini S, et al. Immunoexpression of Ezrin and CD44 in patients with osteosarcoma. J Pediatr Hematol Oncol. 2010; 32:e213-7.
21. Hosono K, Nishida Y, Knudson W, et al. Hyaluronan oligosaccharides inhibit tumorigenicity of osteosarcoma cell lines MG-63 and LM-8 in vitro and in vivo via perturbation of hyaluronan-rich pericellular matrix of the cells. Am J Pathol. 2007; 171:274-86.
22. Arai E, Nishida Y, Wasa J, et al. Inhibition of hyaluronan retention by 4-methylumbelliferone suppresses osteosarcoma cells in vitro and lung metastasis in vivo. Br J Cancer. 2011.
23. Salzer-Kuntschik M, Delling G, Beron G, et al. Morphological grades of regression in osteosarcoma after polychemotherapy - study COSS 80. J Cancer Res Clin Oncol. 1983; 106 Suppl:21-4.
24. Kononen J, Bubendorf L, Kallioniemi A, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med. 1998; 4:844-7.
25. Sabile AA, Arlt MJ, Muff R, et al. Cyr61 expression in Osteosarcoma indicates poor prognosis and promotes intratibial growth and lung metastasis in mice. J Bone Miner Res. 2011.
26. Ruifrok AC, Johnston DA. Quantification of histochemical staining by color deconvolution. Anal Quant Cytol Histol. 2001; 23:291-9.

27. Arlt MJ, Banke IJ, Walters DK, et al. LacZ transgene expression in the subcutaneous Dunn/LM8 osteosarcoma mouse model allows for the identification of micrometastasis. J Orthop Res. 2011.
28. Steinmann P, Walters DK, MJ EA, et al. Antimetastatic activity of honokiol in osteosarcoma. Cancer. 2011.
29. Clark JC, Dass CR, Choong PF. A review of clinical and molecular prognostic factors in osteosarcoma. J Cancer Res Clin Oncol. 2008; 134:281-97.
30. Siclari VA, Qin L. Targeting the osteosarcoma cancer stem cell. J Orthop Surg Res. 2010; 5:78.
31. Brown RL, Reinke LM, Damerow MS, et al. CD44 splice isoform switching in human and mouse epithelium is essential for epithelial-mesenchymal transition and breast cancer progression. J Clin Invest. 2011; 121:1064-74.
32. Xu-Dong S, Zan S, Shui-er Z, et al. Expression of Ezrin correlates with lung metastasis in Chinese patients with osteosarcoma. Clin Invest Med. 2009; 32:E180-8.
33. Rabin K, Man TK, Lau CC. Personalized care of pediatric cancer patients. Nestle Nutr Workshop Ser Pediatr Program. 2008; 62:173-85; discussion 85-8.
34. Toole BP, Slomiany MG. Hyaluronan: a constitutive regulator of chemoresistance and malignancy in cancer cells. Seminars in cancer biology. 2008; 18:244-50.
35. Slomiany MG, Dai L, Bomar PA, et al. Abrogating drug resistance in malignant peripheral nerve sheath tumors by disrupting hyaluronan-CD44 interactions with small hyaluronan oligosaccharides. Cancer Res. 2009; 69:4992-8.

Figure legends

Fig. 1. Kaplan-Meier analyses of osteosarcoma tissue microarray immunostained with pan-CD44 antibodies. (*A*) Representative images of tumor tissue microarray sections showing non-detectable (left), moderate (middle) and intense (right) CD44 immunostaining. (*B*) Event-free survival of responders (Salzer-Kuntschik et al. [23], grade I-III) or of non-responders (grade IV-VI) to neoadjuvant chemotherapy. (*C*) Overall survival of patients with non-detectable (CD44 neg) or with moderate or intense (CD44 pos) immunostaining of tumor tissue. Overall (*D*) and event-free (*E*) survival of non-responders to neoadjuvant therapy with CD44 neg or CD44 pos tumors. (*F*) Overall survival of patients with metastases and CD44 neg or CD44 pos tumors.

Fig. 2. Overexpression of CD44s in SaOS-2 cells increases the adhesion to HA, promotes cell migration and enhances cisplatin chemoresistance, but does not affect proliferation. (*A*) SaOS-2 cells were retrovirally transduced with empty vector (EV) or with constructs encoding CD44s or HA binding-defective CD44s R41A, and the expression of respective proteins was confirmed on Western blots of cell extracts analyzed with CD44 antibody Hermes3 (H3), V5 antibody and antibody to GAPDH (protein loading control). (*B*) Adhesion (% of seeded cells) to immobilized hyaluronan within 30 min of EV-transduced or of CD44s - or CD44s R41A expressing cells. (*C*) Adhesion (% of seeded cells) of CD44s expressing cells to hyaluronan in the absence (-H1) or in the presence (+H1) of the CD44-blocking Hermes1 antibody or of control IgG. Transwell migration (% of seeded cells) (*D*), proliferation (*E*) of EV-transduced or of CD44s or CD44s R41A expressing cells and cisplatin cytotoxicity (*F*) in respective cell lines determined in a WST-1 assay. IC_{50}, half-maximal growth inhibitory concentrations of cisplatin. Values in (*B*) - (*F*) are expressed as means ± SEM of at least three independent experiments; *, $p < 0.05$.

Fig. 3. Overexpression of CD44s in SaOS-2 cells enhances intratibial osteoblastic primary tumor growth in SCID mice in a HA-dependent manner. (*A*) Representative X-ray images of tumor-bearing legs taken on indicated days after intratibial injection of cells transduced with empty vector (EV) (11 mice) or cells expressing CD44s (8 mice) or CD44s R41A (9 mice). (*B*) Mean (± SEM) primary tumor volume in respective mice 12 weeks after tumor cell injection. *, $p < 0.05$.

Fig. 4. CD44s overexpression in SaOS-2 cells enhances pulmonary metastasis of intratibial primary tumors in a HA-dependent manner in SCID mice. (*A*) Representative images of blue X-gal stained metastatic nodules in the lungs of mice bearing tumors derived from empty vector (EV)-transduced or CD44s - or CD44s R41A expressing cells. Bars, 250 μm. Quantification of pulmonary macrometastases (*B*) and micrometastases (*C*) on lung whole mounts prepared after sacrifice in week 12 after tumor cell injection. Values are the mean ± SEM; *, $p < 0.05$.

Table 1: Clinical characteristics of osteosarcoma patients

	n=53	%
Gender		
Male	34	64
Female	19	36
Age		
<10	10	19
10 to 19	26	49
20 to 29	11	21
30 to 39	2	4
40 to 49	2	4
50 to 59	2	4
Tumor type		
Osteoblastic	38	72
Chondroblastic	7	13
Fibroblastic	4	8
Teleangiectatic	4	8
Anatomic site		
Extremities	46	87
Spine and pelvis	6	11
Face	1	2
Chemotherapy response		
Responders (S-K-I-III)	30	57
Non-responders (S-K-IV-VI)	23	43
Salzer-Kuntschik I	6	11
Salzer-Kuntschik II	13	25
Salzer-Kuntschik III	11	21
Salzer-Kuntschik IV	11	21
Salzer-Kuntschik V	9	17
Salzer-Kuntschik VI	3	6
Metastasis		
No metastasis	34	64
Total metastasis	19	36
Metastasis at diagnosis	2	4
Metastasis after diagnosis	17	32

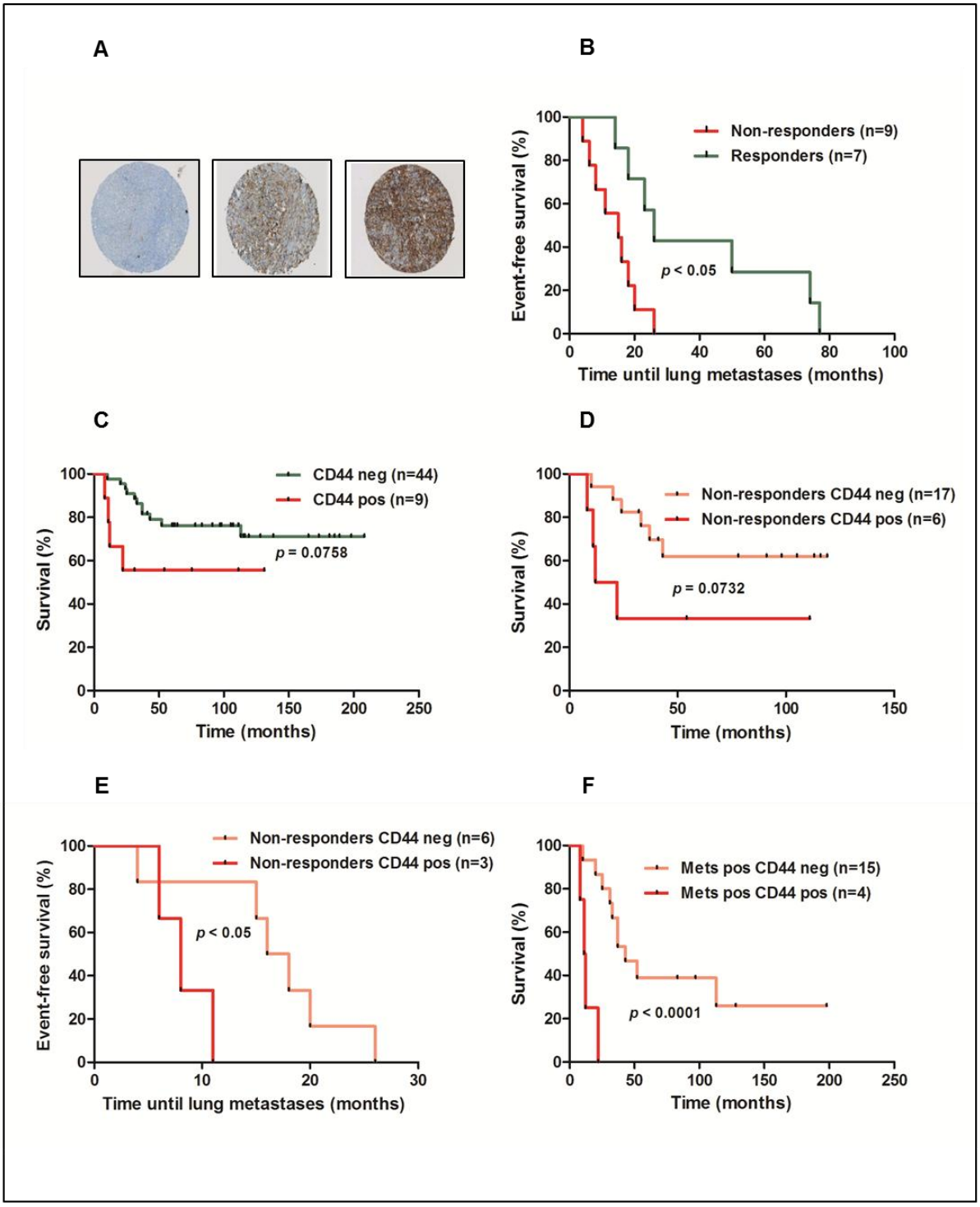

A
B
Non-responders (n=9)
Responders (n=7)
p < 0.05
Event-free survival (%)
Time until lung metastases (months)
C
CD44 neg (n=44)
CD44 pos (n=9)
p = 0.0758
Survival (%)
Time (months)
D
Non-responders CD44 neg (n=17)
Non-responders CD44 pos (n=6)
p = 0.0732
Survival (%)
Time (months)
E
Non-responders CD44 neg (n=6)
Non-responders CD44 pos (n=3)
p < 0.05
Event-free survival (%)
Time until lung metastases (months)
F
Mets pos CD44 neg (n=15)
Mets pos CD44 pos (n=4)
p < 0.0001
Survival (%)
Time (months)

Fig. 1.

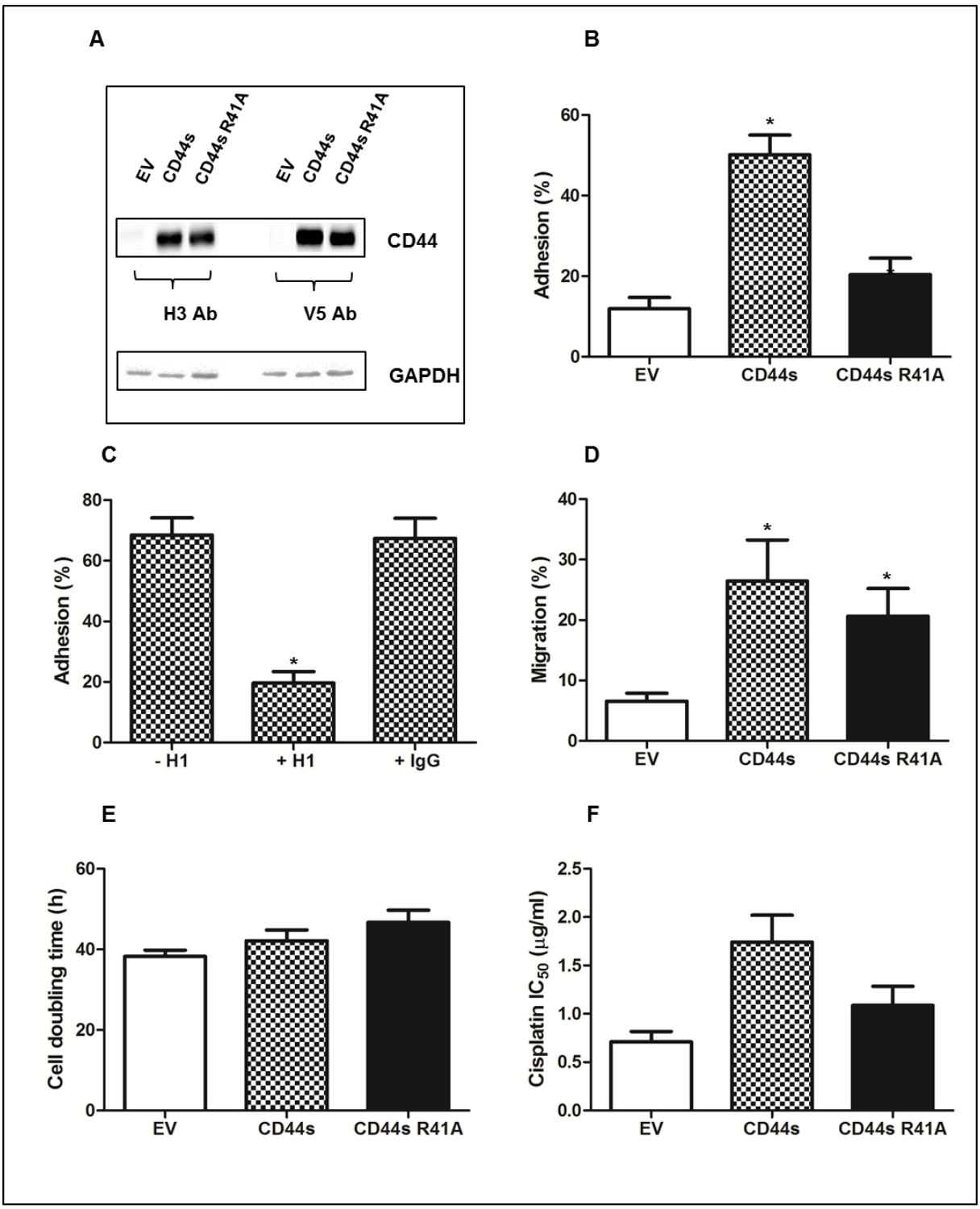

A
EV
CD44s
CD44s R41A
EV
CD44s
CD44s R41A
CD44
H3 Ab
V5 Ab
GAPDH
B
Adhesion (%)
60
40
20
0
*
EV
CD44s
CD44s R41A
C
Adhesion (%)
80
60
40
20
0
*
- H1
+ H1
+ IgG
D
Migration (%)
40
30
20
10
0
*
*
EV
CD44s
CD44s R41A
E
Cell doubling time (h)
60
40
20
0
EV
CD44s
CD44s R41A
F
Cisplatin IC50 (μg/ml)
2.5
2.0
1.5
1.0
0.5
0.0
EV
CD44s
CD44s R41A

Fig. 2.

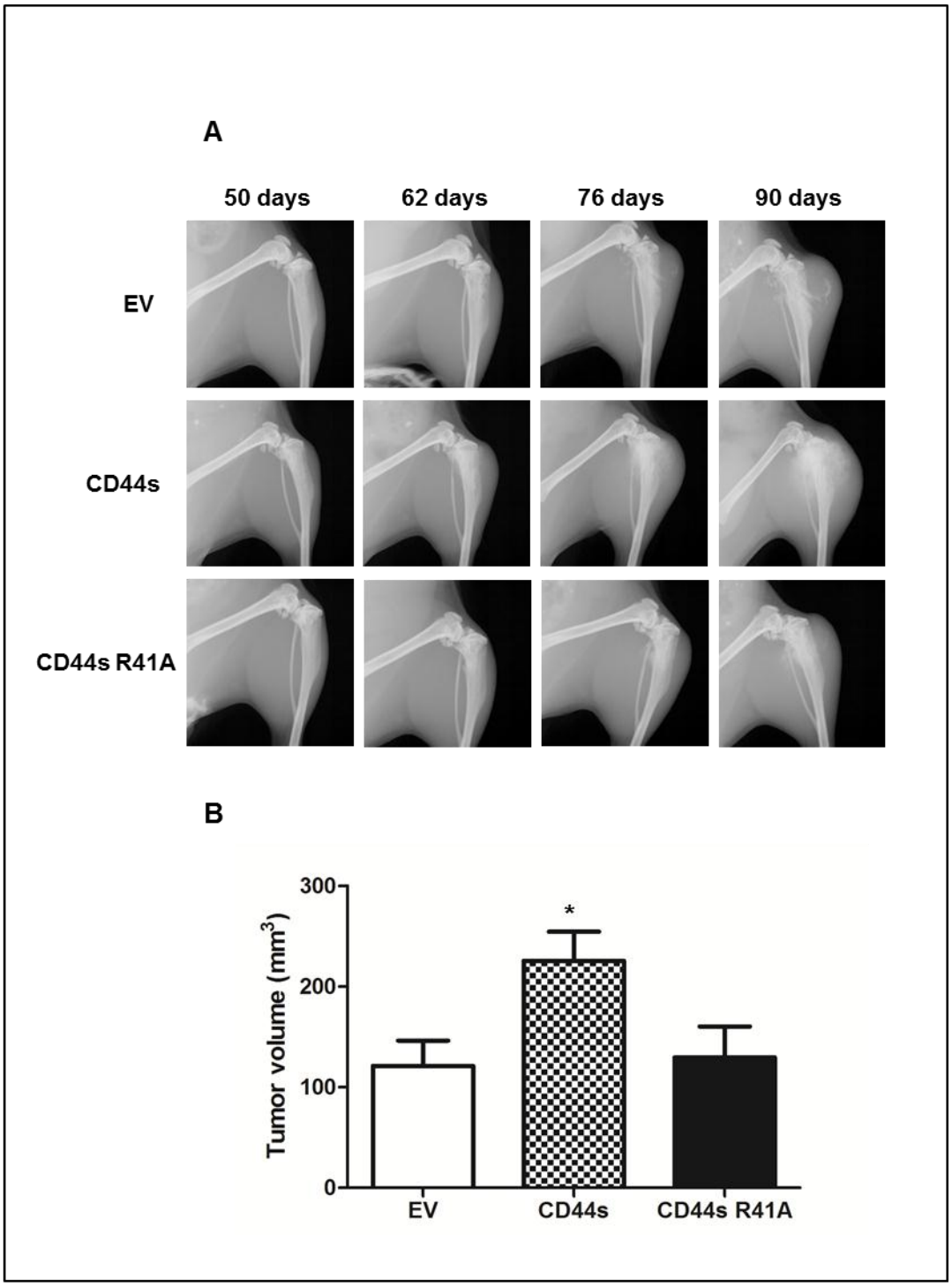

A
50 days
62 days
76 days
90 days
EV
CD44s
CD44s R41A
B
Tumor volume (mm3)
300
200
100
0
*
EV
CD44s
CD44s R41A

Fig. 3.

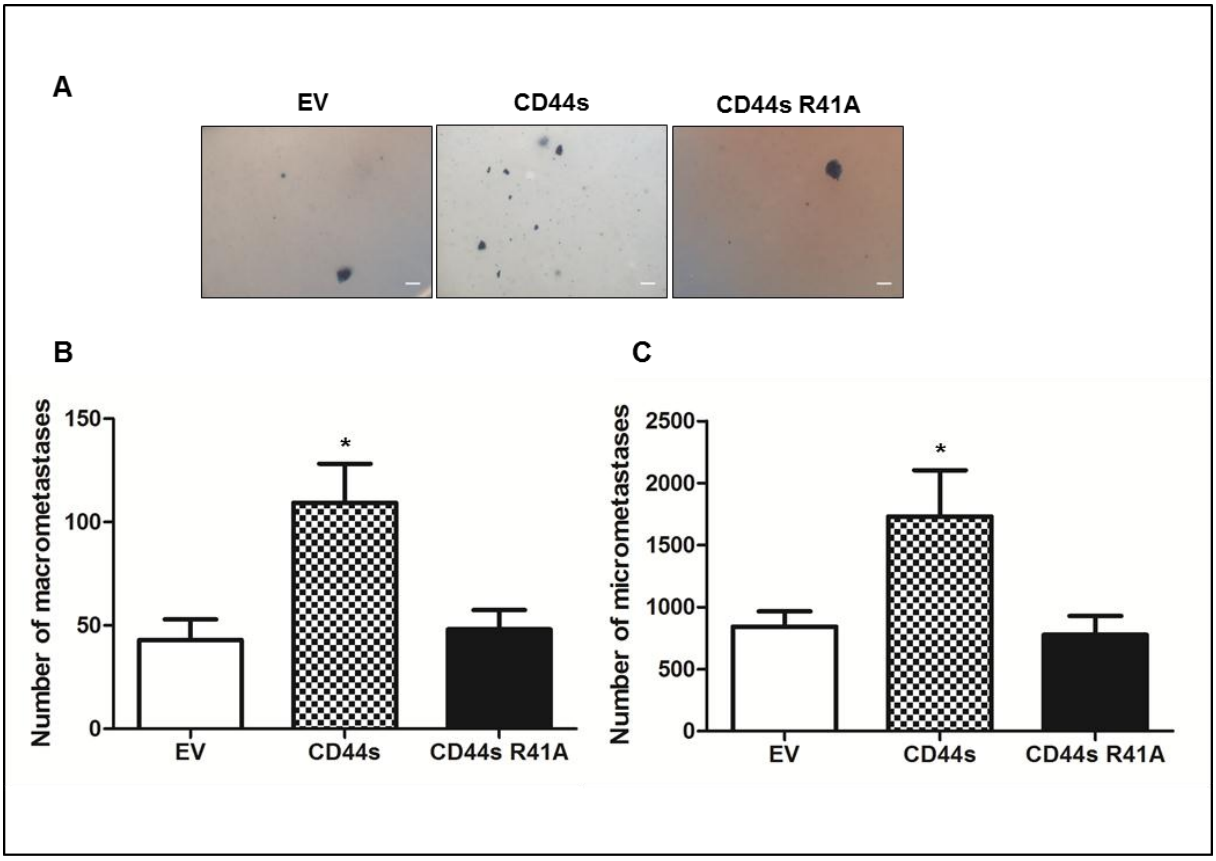

A
EV
CD44s
CD44s R41A
B
Number of macrometastases
150
100
50
0
*
EV
CD44s
CD44s R41A
C
Number of micrometastases
2500
2000
1500
1000
500
0
*
EV
CD44s
CD44s R41A

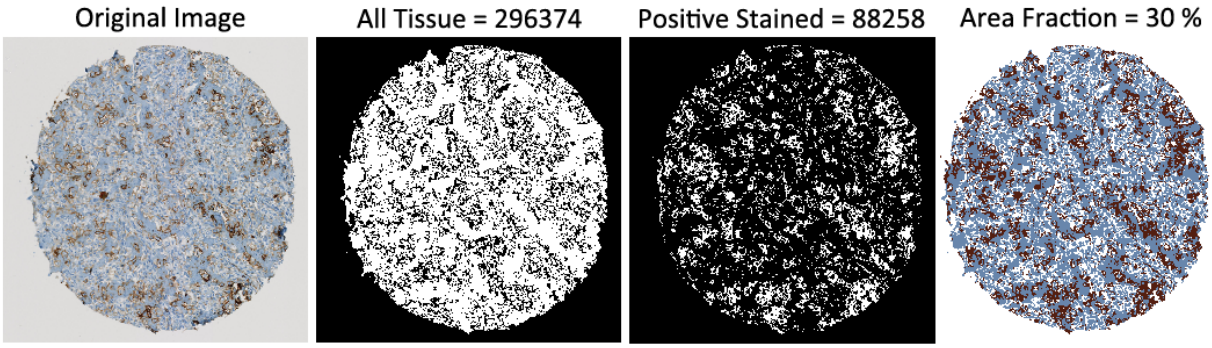
Fig. 4.

Supplemental data

Table 1. Tissue microarray grading method

	0 – 10 % staining	**11 – 50 % staining**	**51 – 100 % staining**
Weak staining	1	1	1
Moderate staining	1	2	2
Strong staining	1	2	3

Fig. 1. Example of positive staining area percentage calculation



4.2 Manuscript 2:

CD44 Acts as Metastasis Suppressor in an Orthotopic Xenograft Osteosarcoma Mouse Model

Ana Gvozdenovic,[1] Matthias JE Arlt,[1] Carmen Campanile,[1] Patrick Brennecke,[1] Knut Husmann, [1] Walter Born,[1] Roman Muff,[1] and Bruno Fuchs[1]

[1]Laboratory for Orthopedic Research, Department of Orthopedics, Balgrist University Hospital, Zurich, Switzerland

Address correspondence to: Bruno Fuchs, MD, PhD, Laboratory of Orthopedic Research, Balgrist University Hospital, Forchstrasse 340, CH-8008 Zurich, Switzerland. E-mail: bfuchs@research.balgrist.ch

Disclosures

All the authors state that they have no conflicts of interest.

Abstract

Osteosarcoma (OS) is the most common primary malignant bone cancer in children and adolescents with a high propensity for lung metastasis**.** Therefore, it is of great importance to identify molecular markers leading to increased metastatic potential in order to devise more effective therapeutic strategies that suppress metastasis, the major cause of death in OS**.** CD44, the principal receptor for the extracellular matrix component hyaluronan (HA), is frequently found overexpressed in tumour cells and has been implicated in metastatic spread in various cancer types. Here, we investigated the effects of stable shRNA-mediated CD44 down-regulation on *in vitro* and *in vivo* metastatic properties of the highly metastatic human 143-B OS cell line. *In vitro*, CD44 knockdown resulted in a 73 % decrease in the adhesion to HA, a 57 % decrease in the migration rate in a trans-filter migration assay, and a 28 % decrease in the cells' capacity for anchorage-independent growth in soft agar, implicating that CD44 expression contributes the metastatic activity of 143-B cells. However, making use of an orthotopic xenograft OS mouse model, we demonstrated that reduced CD44 expression facilitated primary tumor growth and formation of pulmonary metastasis. The enhanced malignant phenotype was associated with decreased adhesion to HA and reduced expression of the tumor suppressor merlin *in vivo*. In conclusion, our study identified CD44 as a metastasis suppressor in this particular experimental OS.

Introduction

Osteosarcoma (OS) is the most common primary malignant bone cancer in children and adolescents, characterized by the presence of spindle-like tumor cells which produce immature bone or osteoid. The overall incidence is three patients/million/year with the median peak at the age of 16 [1]. OS has a high propensity for metastasis to the lung and bones and already 20% of patients have detectable metastases at the time of diagnosis, while 80% of patients who initially present with localized disease subsequently develop metastases [2,3]. Significant clinical improvements over the past several decades through the use of combination chemotherapy and surgery have led to a dramatic increase in the survival of patients with localized disease. However, patients with metastatic or recurrent disease continue to have a very poor prognosis, with <20% long term survival [3] . Therefore, it is of great importance to elucidate the molecular mechanisms leading to increased metastatic potential. Understanding the basic biology of OS progression will enable us to devise novel strategies for treatment that suppress metastasis, the major cause of death in OS, and that will finally improve the survival of these patients.

CD44 has been described to be associated with increased metastatic spread in different types of cancer [4]. It is a cell-cell and cell-matrix adhesion molecule and the principal receptor for hyaluronan (HA), a major component of the extracellular matrix [5]. CD44 is a polymorphic transmembrane glycoprotein with various biological functions in development, inflammation, haematopoiesis, wound healing, immune response and tumor progression [6]. The wide range of CD44 activities is mainly due to post-translational modifications and the existence of multiple isoforms, generated by the insertion of variable regions in the extracellular domain through alternative splicing [7]. Over the last decade, numerous publications have addressed the relevance of CD44 isoforms as prognostic factors for human

cancers. It appears that the CD44 expression and the occurrence of metastasis are directly or inversely related depending on the organ of origin and the pattern of CD44 isoforms expression. Studies have shown up-regulated expression of CD44 variants in many human tumors, including gastric cancer, pancreatic cancer, lung and renal cell cancer. [8,9,10,11]. Several reports point to the importance of the standard form of CD44 (CD44s), without the variant exons, in the tumor progression [12]. However, in other tumor types, namely neuroblastoma and prostate cancer, the lack of CD44 expression (both the standard and the splice variants) correlates with poor prognosis [13,14]. CD44 even acts as a metastasis-suppressor gene in prostate carcinoma cells [6,15].

Little is known about the contribution of CD44 to OS progression and metastasis. Two immunohistochemical studies of osteosarcoma tissue specimens came to different conclusions. Kim et al. reported that overexpression of CD44v5 correlated significantly with metastasis and lower survival rates [16]. On the other hand, in the tumor samples analyzed by Kuryu et al. a correlation between the overexpression of CD44 isoforms containing variant v6 and poor prognosis was found [17]. In both studies total CD44 expression was not correlated with prognosis.

Few studies with established OS cell lines indicated the relevance of HA in tumor progression. Hosono et al. showed the tumorigenicity of the OS cell lines MG63 and LM8 *in vitro* and *in vivo* was inhibited by HA oligosaccharides via perturbation of HA-rich pericellular matrix of the cells [18]. Study performed by Nishida et al. showed an inhibition of HA retention and tumorigenicity of MG63 OS cells upon antisense inhibition of HA synthase (HAS)-2 [19]. HAS-3 derived HA enhanced OS cell activities required for metastasis, such as proliferation, invasion, and degradation of extracellular matrix [20].

In the present work, we investigated the effects of CD44 down-regulation in a highly metastatic osteosarcoma cell line 143-B on its' metastatic behavior *in vitro* as well as the effects on tumor growth and metastasis formation *in vivo* in a xenograft orthotopic mouse model of osteosarcoma.

Materials and Methods

Cell culture and transduction

143-B (CRL-8303) cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). The cells were cultured in DMEM (4.5 g/l glucose)/HamF12 (1:1) medium (Invitrogen; Carlsbad, CA) supplemented with 10% heat-inactivated FCS (GIBCO, Basel, Switzerland). Cells were cultured at 37℃ in a humidified atmosphere of 5% CO_2/95% air. 143-B cells stably overexpressing *LacZ* gene (Husmann et al., submitted) were used for stable knockdown of CD44 using a retroviral approach. Specific shRNA against CD44 (shCD44) and a non-targeting control shRNA (Ctrl shRNA) in pSirenRetroQ vector (Clontech; Paolo Alto, CA) were kindly provided by Prof. Ivan Stamenkovic (Lausanne, Switzerland) [21]. Retroviral particles containing shCD44, Ctrl shRNA and pSirenRetroQ empty vector (EV) were produced in HEK293-T cells according to a modified protocol of Mitta et al. as described in Arlt et al. [22]. 143-B/*LacZ* cells were infected by 48h incubation with virus-containing medium supplemented with 8 µg/ml polybrene. Cells were subsequently maintained in cell culture medium containing 2 µg/ml puromycin (Invitrogen). Selection revealed 143-B EV, 143-B shCD44 and 143-B Ctrl shRNA. Prior to animal experiments the population of cells with silenced CD44 was enriched by incubation on HA coated plates (100 µg/cm^2; Sigma Aldrich, St. Luis, MO) for 10 min at 37℃. Supernatant containing the non-adhered cells was collected.

Immunocytochemistry

Cells were allowed to grow to subconfluency on glass microscope cover slips in 24-well plates. After washing with PBS, the cells were fixed in 4% formalin in PBS for 20 min at room temperature (RT). DMEM/F12 (1:1) containing 0.1% BSA was used for blocking for 30 min at RT. Cells were then incubated with the primary pan anti CD44 antibody (Hermes3; 2 µg/ml in blocking medium, a kind gift from Dr. S. Jalkanen, Turku, Finland) for 2h at RT. After extensive washing secondary anti-mouse antibody (Alexa Fluor 546; Invitrogen) at a final dilution 1:200 was added to the cells and incubated for 30 min in the dark. The coverslips were washed with PBS, dipped in H_2O, mounted in Immomount (ThermoScientific; Waltham, MA) and examined by fluorescence microscopy using the Zeiss Observer.Z1 inverted microscope equipped with an appropriate filter block (Ziess 43) (Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

Western blot analysis

Cells were lysed on a carrousel at 4℃ in lysis buffer containing 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF) and 10 mg/ml aprotinin. The cellular debris was removed by centrifugation at 13000 rpm, 4℃ for 20 min. Equal amounts of proteins were separated by 8% SDS-PAGE. Following electrophoresis gels were blotted onto Hybond-ECL membranes (GE Healthcare, UK). For detection of CD44 and GAPDH mouse monoclonal antibody Hermes3 at concentration 1 µg/ml, and rabbit polyclonal anti-GAPDH (Santa Cruz Biotechnologies, CA; 1:3000) antibodies were used. HRP-conjugated secondary antibodies were purchased at Santa Cruz Biotechnologies. The peroxidase-conjugated antibodies were visualized by chemoluminescence with the Immobilon chemoluminescence substrate (Millipore, Billerica, MA) using the VersaDoc™ Imaging System (Bio-Rad; Hercules, CA).

Adhesion assay

Adhesion assay was performed in 96-well plates coated with 333 µg/cm^2 of high molecular weight HA (HMW-HA; Sigma-Aldrich) over night at 4℃, washed with PBS and blocked with heat-denatured (HD) 1% BSA. Non-coated wells or wells coated only with HD-BSA were used as controls. Subconfluent cells were detached with accutase (Sigma-Aldrich) and 10^4 cells per well were seeded in triplicates and allowed to adhere for 30 min at 37℃. Non-adherent cells were removed by washing and adherent cells were subsequently fixed with 10% formalin in PBS for 15 min at RT and stained with 0.05% crystal violet in H_2O for 15 min at RT. Photos were taken with the AxioCam MRm camera connected to the Zeiss Observer.Z1 inverted microscope at 4x magnification corresponding to an area of 3.6 mm^2 and the number of adherent cells was determined by ImageJ software. The total number of adherent cells per well was calculated and the percentage of adherent cells was obtained by dividing the number of adherent cells with the total number of seeded cells and multiplying with 100. The calculated percentage of adherent EV cells was set as 100% and the results are presented in relation to EV cells. The experiments were repeated three times.

Migration assay

The transwell migration assay was conducted as reported recently [22]. Briefly, 5-10 x 10^3 cells were allowed to migrate through cell culture inserts (Becton Dickinson, San Jose, CA) with 8 µm porous filters in 24-well plates for 6h at37℃. After incubation non-migrated cells on the upper side of the insert were removed by wiping with a cotton swab. Migrated cells on the lower side of the filters were fixed with 10% formalin, permeabilised with 50 µM digitonin (Calbiochem; Switzerland) and stained with 300 nM Picogreen in PBS (Invitrogen; 1:200) for 15 min at RT. Three pictures per insert (two inserts per cell line) were taken the AxioCam MRm camera connected to the Zeiss Observer.Z1 inverted microscope equipped

with an appropriate filter block for blue excitation at a 10x magnification corresponding to an area of 0.58 mm^2. The number of cells that migrated to the lower side of the polycarbonate filters was determined using the ImageJ software, and the results are presented as described for the adhesion assay. The experiments were performed at least three times.

In vitro cell proliferation assay

Subconfluent cells in the logarithmic growth phase were trypsinized and 5 x 10^4 cells were seeded in triplicates in 12.5 cm^2 flasks in 2.5 ml of cell culture medium and allowed to grow for 1 to 5 days. Cells were detached by trypsin every 24h and cell number was determined by counting in the Neubauer chamber. The equation $N=N_o\ e^{kt}$ was used to calculate the doubling time during logarithmic growth

Soft agar colony formation assay

To form the bottom agar layer 1.5 ml of 0.5% DNA grade agarose (Promega, Madison, WI) in cell culture medium were added per well in 6-well plates**.** The plates were kept at 4℃ before use. 2 x 10^4 cells were mixed in 1.5 ml of 0.35% agarose in cell culture medium and added on top of the bottom agar layer. After 24h at 37℃ 2 ml per well of cell culture medium with PSA was added. The medium was replaced every 3 days and the cells were cultured for 14 days. Colonies were stained with 2 ml 0.005% crystal violet overnight at 4℃. Three photos per well were taken with the AxioCam MRm camera connected to the Zeiss Observer.Z1 inverted microscope at 4x magnification. ImageJ software was used for determining the number and size distribution of colonies. The number of colonies formed by EV cells was set as 100%. The experiments were repeated four times in triplicates.

Intratibial xenograft mouse OS model

SCID/CB17 immunocompromised mice were obtained from Charles River Laboratories (Sulzfeld, Germany) at the age of 8 weeks, at least 14 days before the start of the experiment. The animal experiments were approved by the authorities of Kanton Zürich and were conducted in accordance with the guidelines of the "Schweizer Bundesamt für Veterinärwesen". On day 0 of the experiment 2 x 10^5 of 143-B cells (engineered as described) in 10 µl of PBS/0.05% EDTA containing Matrigel (Becton-Dickinson; Franklin Lake, NJ) were injected intratibially. Following the injection, mice health condition was closely monitored. The tumor length and width were measured with a caliper and the X-ray detection of osteolytic lesions was performed with MX-20 DC Digital Radiography System (Faxitron X-Ray Corporation, Lincolnshire, IL) once per week. Tumor volume was calculated according to the formula V= length x width^2/2. The mice were sacrificed 21 days after tumor cell injection and in situ lung perfusion was conducted as described [22]. As the cells used expressed *LacZ* gene, visualization of tumor cells within mouse tissue was facilitated. Organs were fixed in 2% formaldehyde for 30 min at RT, washed three times with PBS and incubated in 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) staining solution for at least 3 hours as described [23,24]. The indigo-blue stained metastases on the lung surface were counted under the microscope. The animal experiments were carried out three times. Representative experiment is shown.

Immunohistochemistry

Tumors and lungs previously fixed in 4 % formaldehyde were dehydrated through 70%, 96%, 100% ethanol and xylene and then embedded in paraffin (Applied Biosystems, Foster City, CA). Sections (6 µm) were cut and mounted onto slides. The sections were then deparaffinized and rehydrated and the antigen retrieval was performed by heating in 0.1 M citrate buffer (pH 5.8). The endogenous peroxidase was blocked by incubation with 3% H_2O_2

for 10 min atRT. The unspecific binding was blocked by incubation with 10% goat serum (Vector Laboratories; Burligame, CA) containing 0.1% Tween (Sigma Aldrich) in Tris buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.4) for 1h at RT. Primary antibodies anti-CD44 Hermes3 (2 µg/ml, diluted in blocking solution), anti-merlin NF2 (Santa Cruz Biotechnologies; 4µg/ml) and anti-Ki67 (Abcam; Cambridge, UK; 4 µg/ml) were then applied to the slides for 1h at RT. After washing with TBS, secondary biotinylated goat anti-mouse IgG antibody (Vector; 1:200) was applied to the sections for 1h at RT. Omission of primary antibody served as negative control. After washing with TBS, the sections were incubated with avidin-biotin peroxidase (ABC kit; Vector Laboratories) and again washed with TBS. The color was developed with AEC (Dako; Carpinteria, CA). Finally, the slides were briefly counterstained with hematoxylin. Recombinant mouse CD44 Fc chimera (100 µg/ml; R&D Systems, Minneapolis, MN) was used for detection of hyaluronan in tissue sections with the standard protocol, excluding the antigen retrieval step. As negative controls we incubated the slides with hyaluronidase (200 U/ml; Sigma Aldrich) overnight at 37℃ prior to staining or we preincubated CD44 Fc chimera with hyaluronan (1 mg/ml; Sigma Aldrich) before applying it to slides.

Statistical analysis

Differences between means were analyzed by the Student t-test and $p<0.05$ was considered significant. The results are presented as means ± SEM.

Results

CD44 down-regulation in the highly metastatic 143-B OS cell line

In order to study CD44 involvement in the malignant phenotype of 143-B OS cells, we stably knocked-down CD44 in 143-B cell using shRNA through retroviral transfer approach as described in *Materials and Methods* section. Western blot analysis of whole cell lysates demonstrated almost abolished total CD44 expression in 143-B shCD44 cells (Fig. 1A).

The cell surface expression assessed by immunocytochemistry in non-permeabilized cells showed CD44 depletion in cells infected with the CD44 specific shRNA (shCD44), whereas cells infected with the control shRNA (Ctrl shRNA) and the empty vector (EV) displayed high levels of membrane-bound CD44 (Fig. 1B).

CD44 silencing inhibits adhesion to hyaluronan and cell migration

Next we investigated whether CD44 down-regulation modulates the cells' ability to bind to HA, the major ligand of CD44. CD44 knock-down resulted in a 73±7.5% ($p<0.02$) decrease in the adhesion to HA compared to the EV cells. Cells transduced with the Ctrl shRNA showed unaltered adhesion in comparison to EV cells (Fig. 2A).

In order to determine whether CD44 silencing might influence migration capacities of 143-B cells we performed a trans-filter migration assay. We observed a 57±4.2% ($p<0.0001$) decrease in migration in cells with down-regulated CD44 in comparison to the EV cells. In contrast, Ctrl shRNA did not have an impact on the migration rate (Fig. 2B).

CD44 silencing has no effect on *in vitro* growth of adherent cells, but reduces anchorage-independent growth

We examined the potential effect of stable CD44 down-regulation on anchorage-dependent and anchorage-independent cell growth. There was no significant difference in the proliferation on plastic of adherent cells in tissue culture flasks between the cells with manipulated CD44 expression and the control cells, with the calculated doubling times being 19.1±0.6 h for EV cells, 19.9±1.3 h for shCD44 cells and 20.0±0.9 h for Ctrl shRNA cells (Fig. 2C). However, when we assessed the anchorage-independent growth in a soft agar colony formation assay, a 28±6% ($p<0.02$) reduction in the total colony number formed by shCD44 cells compared to control cells was found (Fig. 2D). The colony size was comparable between the cell lines (not shown).

Reduced CD44 expression facilitates primary tumor growth and lung metastases formation in a xenograft OS mouse model

The *in vitro* experiments point to important roles of CD44 in several cellular processes contributing to the metastatic activity of 143-B cells. Therefore, we studied how CD44 down-regulation affects primary tumor growth and metastases progression in a mouse model. 143-B cells with suppressed CD44, EV or non-targeting Ctrl shRNA were injected intratibially into SCID/CB17 mice. Osteolytic lesions, that are a characteristic feature for 143-B cells, could be detected by X-ray starting at day 14 after tumor cell injection in all three groups of experimental animals. No dramatic differences in the extent of osteolysis between the groups were found (Fig. 3A). However, CD44 knock-down significantly increased the mean final primary tumor volume (108±14 mm^3, n=9) in comparison with the group of mice injected with cells carrying only the EV (39±6 mm^3, n=9; $p<0.001$). 143-B Ctrl shRNA cells upon injection into mice gave rise to tumors with the mean final volume of 65±25 mm^3 (n=6; Fig.

3B). The difference in tumor volume between shCD44 xenografts and Ctrl shRNA xenografts did not reach significance, most probably due to the fact that Ctrl shRNA animals exhibited wide heterogeneity in tumor size. Next, we determined the number of metastases on the lung surface visualised after X-Gal staining. Fig. 3C shows representative images of X-Gal stained lungs of animals with EV, Ctrl shRNA and shCD44 bearing tumors. The numbers of metastatic nodules were increased 2-fold and 2.4-fold compared to that of in the EV group ($p<0.05$) and the Ctrl shRNA group ($p<0.05$), respectively (Fig. 3D).

CD44 suppression is maintained *in vivo* on tumor cells containing hyaluronan-rich extracellular matrices

After these unexpected findings regarding the primary tumor growth and metastases formation that are in conflict with the *in vitro* data, we asked whether CD44 suppression is still stable *in vivo*. For this reason, we performed immunohistochemistry with Hermes3 antibody on tumor sections from the three experimental groups. Expression of CD44 was high in both the EV and Ctrl shRNA tumors (Fig. 4A, B). On the other hand, we found almost no CD44 protein expression in tumors formed in mice orthotopically injected with 143-B cells transduced with a specific shRNA against CD44 (Fig. 4C). The pulmonary metastases had the same CD44 expression pattern as the primary tumors (Fig. 4G-I).

The relevance of cell-cell and cell-matrix adhesion and their involvement in each subsequent phase of the metastatic journey has been well established and adhesion is considered as the fundamental molecular effector mechanism upon which a metastatic cell relies [25]. We propose that the detachment of OS cancer cells from the primary tumor mass is supported by loss of intercellular and cell-matrix contacts in 143-B shCD44 cells through down-regulation of CD44. Therefore, we used immunostaining with recombinant CD44-Fc chimera with the aim to detect hyaluronan in primary tumors that originate from manipulated

143-B cells. Irrespective of CD44 presence or absence on tumor cells, hyaluronan was found to be abundantly expressed in the extracellular matrices of these cells (Fig. 4 D-F). Decreased adhesion of 143-B shCD44 cells to hyaluronan-rich matrices may facilitate their spread beyond the primary tumor site, finally leading to elevated metastatic ability. Indeed, Ki67 staining of proliferating tumor cells showed a specific structure of primary tumors and lung metastases in mice bearing tumors depleted of CD44. As opposed to EV and Ctrl shRNA carrying 143-B cells that are compactly organized and in close cell contact with each other (Fig. 5A, B, D, E), 143-B shCD44 cells show a low-adhesive phenotype with wide inter-cellular spaces (Fig. 5C, F). This lack of cell-cell junctions might simplify cell motility and dissemination.

Merlin expression *in vivo* is suppressed upon CD44 silencing

It has been reported that CD44 interacts with merlin, a tumor and metastasis suppressor. When exposed to hyaluronan CD44 binds through its cytoplasmatic tail to merlin that is subsequently activated to confer growth arrest by contact inhibition [26]. Therefore, we sought to explore merlin protein levels in manipulated 143-B cells. *In vitro,* all three cell lines expressed equal protein amounts of merlin (not shown). In contrast, merlin expression was diminished in primary tumors, as well as in pulmonary metastases in mice bearing shCD44 xenografts when compared to tumor tissue in EV and Ctrl shRNA xenografts (Fig. 5 G-L). The lack of merlin *in vivo* might be correlated with the increase in tumor volume and metastatic capacity of 143-B shCD44 cells.

Discussion

Only a few studies addressed so far the association of CD44 expression with OS progression and metastasis, however obtained results are controversial. Either no correlation

between CD44 gene or protein expression with overall survival of OS patients was observed [27], or OS patients with high CD44 gene expression were found to be more metastases prone in another cohort [28]. Moreover, expression of distinctive CD44 isoforms were found to be correlated with unfavorable prognosis [16,17].

Here, we aimed at determining the role of CD44 in the human 143-B OS cell line with high metastatic activity, which have high endogeneous CD44 expression. Therefore, we down-regulated CD44 expression in 143-B cells and studied its impact both in *in vitro* assays and in an *in vivo* xenograft mouse OS model. *In vitro*, stable shRNA-mediated CD44 knock-down significantly suppressed cell migration and growth in soft agar, implying that CD44 may contribute to the increased metastatic properties of 143-B cells. However, upon intratibial injection into SCID mice, 143-B cells with reduced CD44 expression even enhanced the malignant phenotype when compared to control cells. Mice bearing shCD44 xenografts developed larger primary tumors and had significantly increased number of pulmonary metastases when compared to those in control animals. In contrast to our *in vitro* data, experiments *in vivo* using the orthotopic xenograft mouse model identify CD44 as a metastasis suppressor gene in 143-B cells.

A plausible explanation for the enhanced metastatic activity in cells depleted of CD44 is their decreased adhesion to HA-rich extracellular matrices within primary tumors, consistent with the reduced adhesion to HA we observed *in vitro*. Furthermore, histology of tumor tissue revealed a low adhesive structure of 143-B shCD44 cells with wide intercellular gaps, as opposed to control transduced cells. The changes in adhesive properties of shCD44 cells may also have facilitated their mobility and enabled expansion and dissemination beyond the primary tumor site, ultimately leading to elevated metastatic potential. Study performed by Lopez et al. is in good agreement with our concept, in which they report that CD44 loss has a metastasis-promoting effect in a mouse model of spontaneously metastasizing breast cancer

[29]. Moreover, the authors show that CD44/HA interactions inhibit invasion in a three-dimensional *in vitro* invasion assay, suggesting that CD44 engagement with HA is protective against metastasis. Another explanation for the elevated malignant phenotype in 143-B cells with silenced CD44 is the observed loss of merlin protein expression *in vivo* in these cells. Merlin is encoded by neurofibromatosis type 2 (NF2) gene and mutations and deletions of merlin underlie NF2 familial cancer syndrome, characterized by development of schwannomas, meningiomas and ependymomas [30]. As mutations of NF2 were also detected in other cancer types, it is considered as a tumor suppressor gene in a wide variety of tumor cells. Merlin is a multifunctional protein that regulates cell shape, proliferation, survival, motility and invasion. Interestingly, mice heterozygous for a mutation at the NF2 locus (Nf2+/-) are cancer prone, and develop a wide spectrum of tumors, most frequently OS, that display strikingly high metastatic proclivity, unlike the benign tumors in human patients with NF2 syndrome [31]. This study provided experimental support for the association of NF2 loss and elevated metastatic potential. Conversely to the observations in mice, in human OS patient samples NF2 mutations could not be found, whereas merlin protein could be detected, implicating the apparent differences between the mouse and human OS tumorigenesis [32]. Nevertheless, as merlin functions as a tumor and metastasis suppressor, accelerated tumor growth and enhanced ability to form metastases seen in the intratibial OS mouse model presented here may be the consequence of loss of merlin's expression in 143-B shCD44 cells. A recent report showed that the loss of merlin protein observed in breast cancer tissues is a result of proteosomal degradation induced by osteopontin initiated Akt-mediated phosphorylation of merlin [33]. Mechanisms underlying merlin loss in OS cells need yet to be elucidated. Additionally, merlin has been reported to reverse the Ras-induced malignant phenotype [34]. Given the fact that 143-B cells were generated through Ki-Ras transformation [35], we suggest that Ras-driven metastatic behavior is even more pronounced upon loss of

merlin protein expression *in vivo*. Therefore, we cannot exclude the possibility of a cell-type specific effect of CD44 down-regulation on tumor and metastasis formation. However, CD44 may act as a metastasis suppressor by regulating merlin expression or function in a subset of OS where Ras signaling is involved.

In conclusion, the findings of our study implicate CD44 as a negative regulator of metastasis in 143-B OS cells. The apparent discrepancy between *in vitro* and *in vivo* outcomes of CD44 knock-down on tumorigenic and metastatic properties of 143-B cells highlights the essential impact of tumor surroundings on OS progression. CD44 functions as a metastasis suppressor gene in this particular experimental OS, however future studies focusing on the role of different CD44 isoforms in additional established OS cell lines or in primary cells, as well as investigating CD44 expression in large cohorts of human tumor tissue samples, will contribute to the delineation of its' role in OS.

Acknowledgments

We thank Prof. Ivan Stamenkovic (Department of Experimental Pathology, University of Lausanne) for providing CD44 expression constructs and helpful discussion and Dr. S. Jalkanen (Turku, finland) for providing Hermes3 antibody. We thank Dr. med. Vet. Monika Hilbe (Institute of Veterinary Pathology, Zurich) for the help in immunohistochemistry stainings of hyaluronan and we thank Josefine Bertz and Christopher Bühler for excellent technical assistance.

This work was supported in part by the Zurcher Krebsliga (Zurich, Switzerland), the Swiss National Science Foundation (SNF Grant Nr.31003A-120403), the University of Zurich, the Schweizerischer Verein Balgrist (Zurich, Switzerland), the Walter L. & Johanna Wolf Foundation (Zurich, Switzerland), and the Lydia Hochstrasser Stiftung (Zurich, Switzerland).

Author's Contributions

Designed the study: AG, RM, BF, WB; Conducted experiments: AG; Animal work: AG, MJEA, CC, PB; Provided material: KH; Analyzed data: AG; Wrote the manuscript: AG, RM, WB, BF.

References

1. Picci P (2007) Osteosarcoma (osteogenic sarcoma). Orphanet J Rare Dis 2: 6.
2. Link MP, Goorin AM, Miser AW, Green AA, Pratt CB, et al. (1986) The Effect of Adjuvant Chemotherapy on Relapse-Free Survival in Patients with Osteosarcoma of the Extremity. New England Journal of Medicine 314: 1600-1606.
3. Marina N, Gebhardt M, Teot L, Gorlick R (2004) Biology and Therapeutic Advances for Pediatric Osteosarcoma. Oncologist 9: 422-441.
4. Naor D, Nedvetzki S, Golan I, Melnik L, Faitelson Y (2002) CD44 in cancer. Crit Rev Clin Lab Sci 39: 527-579.
5. Aruffo A, Stamenkovic I, Melnick M, Underhill CB, Seed B (1990) CD44 is the principal cell surface receptor for hyaluronate. Cell 61: 1303-1313.
6. Ponta H, Sherman L, Herrlich PA (2003) CD44: from adhesion molecules to signalling regulators. Nat Rev Mol Cell Biol 4: 33-45.
7. Marhaba R, Zoller M (2004) CD44 in cancer progression: adhesion, migration and growth regulation. J Mol Histol 35: 211-231.
8. Heider K-H, Dämmrich J, Skroch-Angel P, Müller-Hermelink H-K, Vollmers HP, et al. (1993) Differential Expression of CD44 Splice Variants in Intestinal- and Diffuse-Type Human Gastric Carcinomas and Normal Gastric Mucosa. Cancer Research 53: 4197-4203.
9. Rall CJN, Rustgi AK (1995) CD44 Isoform Expression in Primary and Metastatic Pancreatic Adenocarcinoma. Cancer Research 55: 1831-1835.
10. Penno MB, August JT, Baylin SB, Mabry M, Linnoila RI, et al. (1994) Expression of CD44 in Human Lung Tumors. Cancer Research 54: 1381-1387.
11. Lim S, Young A, Paner G, Amin M (2008) Prognostic role of CD44 cell adhesion molecule expression in primary and metastatic renal cell carcinoma: a clinicopathologic study of 125 cases. Virchows Archiv 452: 49-55.
12. Cannistra SA, Kansas GS, Niloff J, DeFranzo B, Kim Y, et al. (1993) Binding of Ovarian Cancer Cells to Peritoneal Mesothelium in Vitro Is Partly Mediated by CD44H. Cancer Research 53: 3830-3838.
13. Shtivelman E, Bishop JM (1991) Expression of CD44 is repressed in neuroblastoma cells. pp. 5446-5453.
14. Angelo M. De Marzo CBJSJIEGJM (1998) CD44 and CD44v6 downregulation in clinical prostatic carcinoma: Relation to Gleason grade and cytoarchitecture. Prostate. pp. 162-168.
15. Gao AC, Lou W, Dong J-T, Isaacs JT (1997) CD44 Is a Metastasis Suppressor Gene for Prostatic Cancer Located on Human Chromosome 11p13. pp. 846-849.
16. Kim HS, Park YB, Oh JH, Jeong J, Kim CJ, et al. (2002) Expression of CD44 isoforms correlates with the metastatic potential of osteosarcoma. Clin Orthop Relat Res: 184-190.
17. Kuryu M, Ozaki T, Nishida K, Shibahara M, Kawai A, et al. (1999) Expression of CD44 variants in osteosarcoma. J Cancer Res Clin Oncol 125: 646-652.

18. Hosono K, Nishida Y, Knudson W, Knudson CB, Naruse T, et al. (2007) Hyaluronan oligosaccharides inhibit tumorigenicity of osteosarcoma cell lines MG-63 and LM-8 in vitro and in vivo via perturbation of hyaluronan-rich pericellular matrix of the cells. Am J Pathol 171: 274-286.
19. Nishida Y, Knudson W, Knudson CB, Ishiguro N (2005) Antisense inhibition of hyaluronan synthase-2 in human osteosarcoma cells inhibits hyaluronan retention and tumorigenicity. Exp Cell Res 307: 194-203.
20. Tofuku K, Yokouchi M, Murayama T, Minami S, Komiya S (2006) HAS3-related hyaluronan enhances biological activities necessary for metastasis of osteosarcoma cells. Int J Oncol 29: 175-183.
21. Janiszewska M, De Vito C, Le Bitoux MA, Fusco C, Stamenkovic I (2010) Transportin regulates nuclear import of CD44. J Biol Chem 285: 30548-30557.
22. Arlt MJ, Banke IJ, Walters DK, Puskas GJ, Steinmann P, et al. (2011) LacZ transgene expression in the subcutaneous Dunn/LM8 osteosarcoma mouse model allows for the identification of micrometastasis. J Orthop Res.
23. Arlt M, Kopitz C, Pennington C, Watson KLM, Krell H-W, et al. (2002) Increase in Gelatinase-specificity of Matrix Metalloproteinase Inhibitors Correlates with Antimetastatic Efficacy in a T-Cell Lymphoma Model. Cancer Research 62: 5543-5550.
24. Kruger A, Schirrmacher V, von Hoegen P (1994) Scattered micrometastases visualized at the single-cell level: detection and re-isolation of lacZ-labeled metastasized lymphoma cells. Int J Cancer 58: 275-284.
25. Bacac M, Stamenkovic I (2008) Metastatic cancer cell. Annu Rev Pathol 3: 221-247.
26. Morrison H, Sherman LS, Legg J, Banine F, Isacke C, et al. (2001) The NF2 tumor suppressor gene product, merlin, mediates contact inhibition of growth through interactions with CD44. Genes Dev 15: 968-980.
27. Boldrini E, Peres SV, Morini S, de Camargo B (2010) Immunoexpression of Ezrin and CD44 in patients with osteosarcoma. J Pediatr Hematol Oncol 32: e213-217.
28. Xu-Dong S, Zan S, Shui-er Z, Li-na T, Wen-xi Y, et al. (2009) Expression of Ezrin correlates with lung metastasis in Chinese patients with osteosarcoma. Clin Invest Med 32: E180-188.
29. Lopez JI, Camenisch TD, Stevens MV, Sands BJ, McDonald J, et al. (2005) CD44 attenuates metastatic invasion during breast cancer progression. Cancer Res 65: 6755-6763.
30. Stamenkovic I, Yu Q (2010) Merlin, a "magic" linker between extracellular cues and intracellular signaling pathways that regulate cell motility, proliferation, and survival. Curr Protein Pept Sci 11: 471-484.
31. McClatchey AI, Saotome I, Mercer K, Crowley D, Gusella JF, et al. (1998) Mice heterozygous for a mutation at the Nf2 tumor suppressor locus develop a range of highly metastatic tumors. Genes Dev 12: 1121-1133.
32. Stemmer-Rachamimov AO, Nielsen GP, Rosenberg AE, Louis DN, Jones D, et al. (1998) The NF2 gene and merlin protein in human osteosarcomas. Neurogenetics 2: 73-74.
33. Morrow KA, Das S, Metge BJ, Ye K, Mulekar MS, et al. (2011) Loss of tumor suppressor Merlin in advanced breast cancer is due to post-translational regulation. J Biol Chem 286: 40376-40385.
34. Tikoo A, Varga M, Ramesh V, Gusella J, Maruta H (1994) An anti-Ras function of neurofibromatosis type 2 gene product (NF2/Merlin). J Biol Chem 269: 23387-23390.
35. Rhim JS, Cho HY, Huebner RJ (1975) Non-producer human cells induced by murine sarcoma virus. Int J Cancer 15: 23-29.

Figure Legends

Figure 1. CD44 down-regulation of CD44 expression by shCD44 in 143-B cells. (A) Western blot analysis showed nearly complete abolishment of total CD44 expression in 143-B cells infected with the CD44 specific shRNA (shCD44) compared to the cells infected with the control shRNA (Ctrl shRNA) and the empty vector (EV). GAPDH was used as a loading control. (B) CD44 surface expression, assessed by immunocytochemistry in non-permeabilized cells revealed reduced CD44 membrane expression in 143-B shCD44 cells when compared to that of 143-B EV and 143-B Ctrl shRNA.

Figure 2. *In-vitro* functional assays. CD44 silencing decreases adhesion to HA (A, n=3), migration in the trans-filter migration assay (B, n=6), and the cells' capacity for anchorage-independent growth in soft agar (D, n=4), but proliferation of adherent cells is not affected (C, n=3). Values represent the mean ± SEM; *, $p<0.05$.

Figure 3. Primary tumor growth and lung metastases formation after intratibial injection of transduced 143-B cells into SCID mice. (A) X-ray monitoring of tumor development showed similar extent of osteolytic lesions in all animal groups. (B) Primary tumor volume was determined every 7 days. Mice injected with cells transduced with the specific shRNA against CD44 (shCD44, n=9) developed larger tumors in comparison to the EV (n=9) and Ctrl shRNA (n=6) experimental groups. (B) Representative photos of the lungs with the blue X-gal stained metastases of mice bearing EV, shCD44 and Ctrl shRNA xenografts. (D) CD44 knock-down promotes metastases in the lungs. Values are expressed as mean ± SEM; *, $p<0.05$.

Figure 4. CD44 and HA immunohistochemical analyses of primary tumors and pulmonary metastases. CD44 levels are remarkably diminished in primary tumor (PT) and pulmonary metastases (PM) sections of mice bearing tumors with specific shRNA against CD44 (C, I),

compared to EV (A,G) and Ctrl shRNA bearing animals (B,H). HA is abundantly expressed in primary tumors (E) in all animal groups. Hermes3 antibody and recombinant CD44-Fc chimera were used to detect CD44 and HA on tissue sections, respectively. Nuclei were counterstained with hematoxylin. Bar, 100 µm.

Figure 5. Ki67 and merlin immunohistochemical analyses of primary tumors and pulmonary metastases. Tissue sections of primary tumors (PT) and pulmonary metastases (PM) stained with polyclonal anti-Ki67 antibody revealed dense organisation of closely attached proliferating cells in EV (A,D) and Ctrl shRNA (B,E) xenografts, in contrast to shCD44 xenografts which displayed a loose structure assembled by low adhesive tumor cells (C,F). Primary tumors and pulmonary metastases originating from 143-B lacking CD44 were almost devoid of merlin expression (I,L), compared to CD44 positive tumors and metastases of control mice (G,H,J,K). Bar, 100 µm.

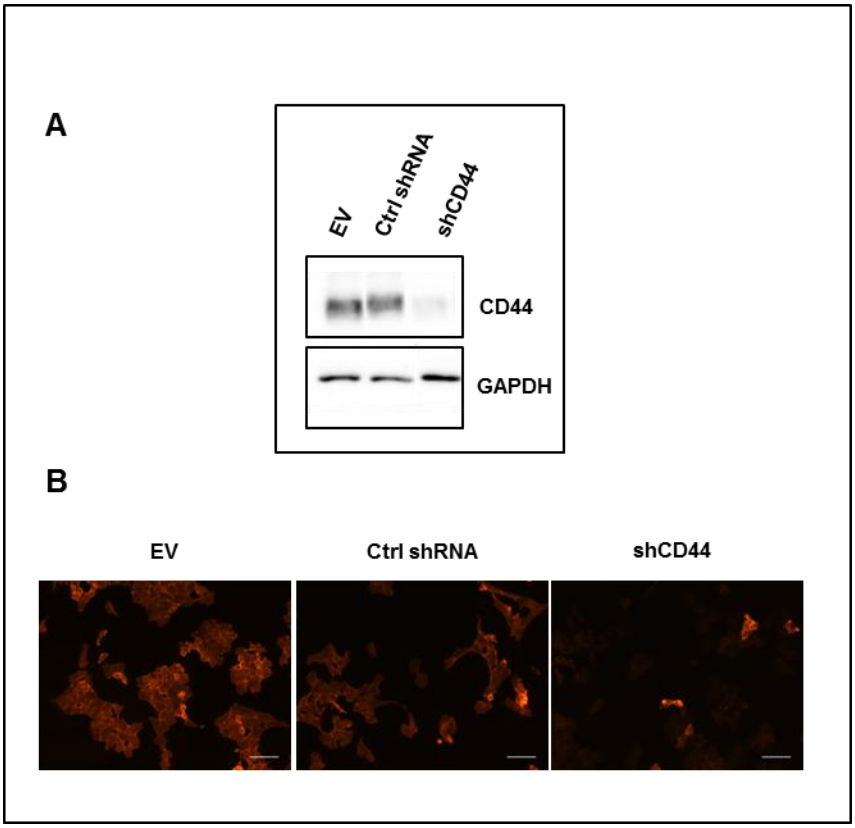


Figure 1.

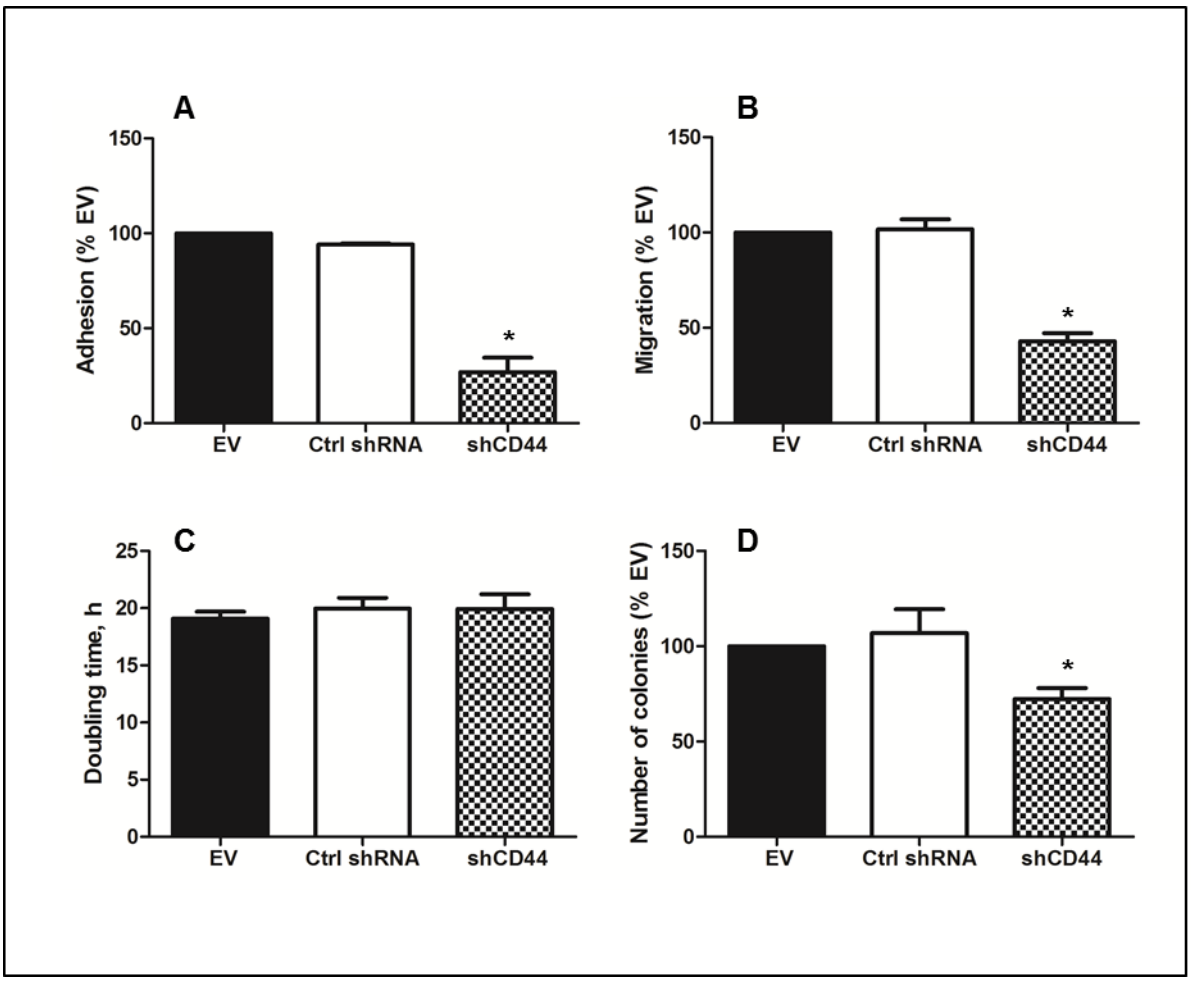

A
Adhesion (% EV)
150
100
50
0
*
EV
Ctrl shRNA
shCD44
B
Migration (% EV)
150
100
50
0
*
EV
Ctrl shRNA
shCD44
C
Doubling time, h
25
20
15
10
5
0
EV
Ctrl shRNA
shCD44
D
Number of colonies (% EV)
150
100
50
0
*
EV
Ctrl shRNA
shCD44

Figure 2.

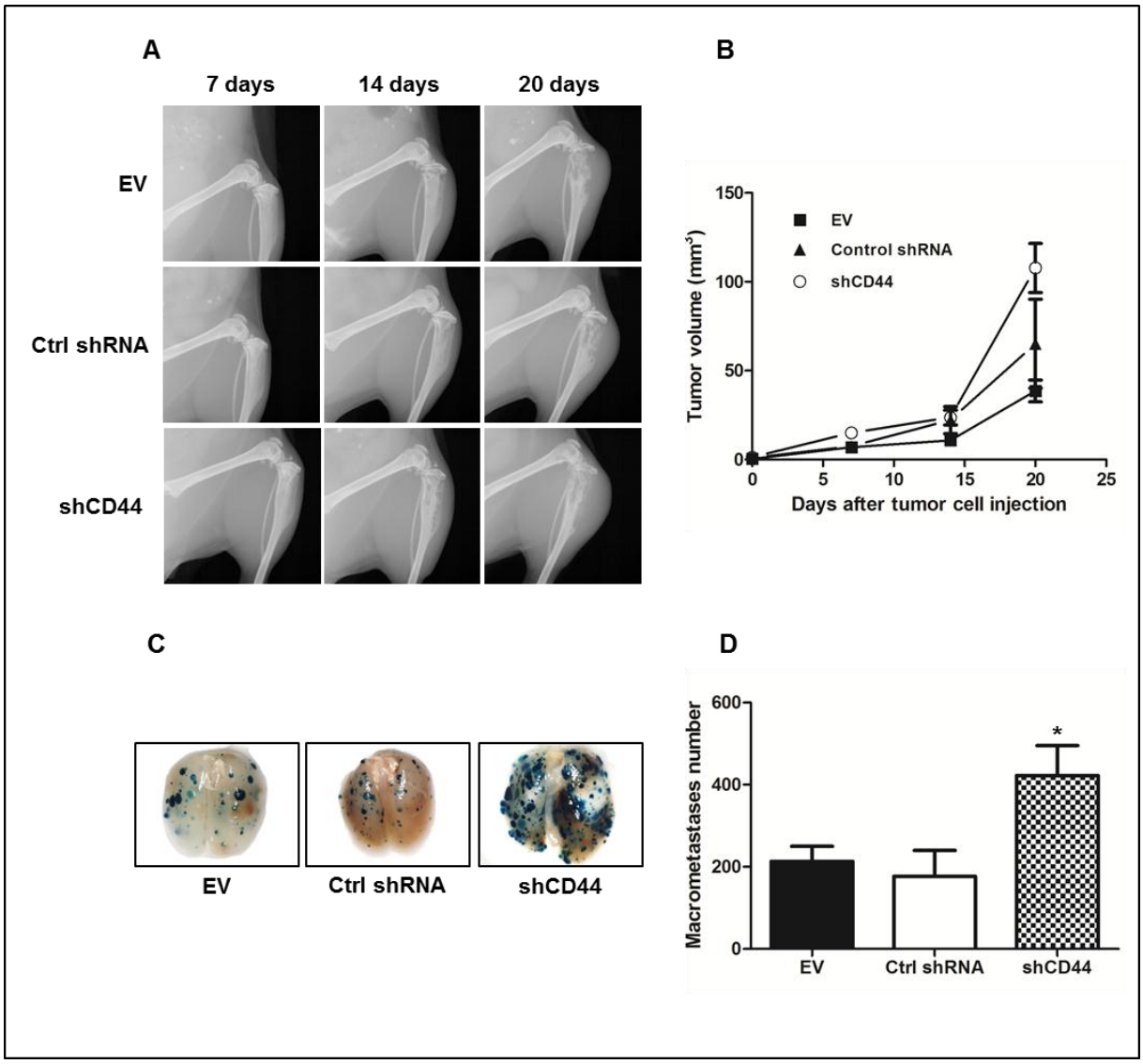

A
7 days
14 days
20 days
EV
Ctrl shRNA
shCD44
B
EV
Control shRNA
shCD44
Tumor volume (mm³)
Days after tumor cell injection
C
EV
Ctrl shRNA
shCD44
D
Macrometastases number
EV
Ctrl shRNA
shCD44

Figure 3.

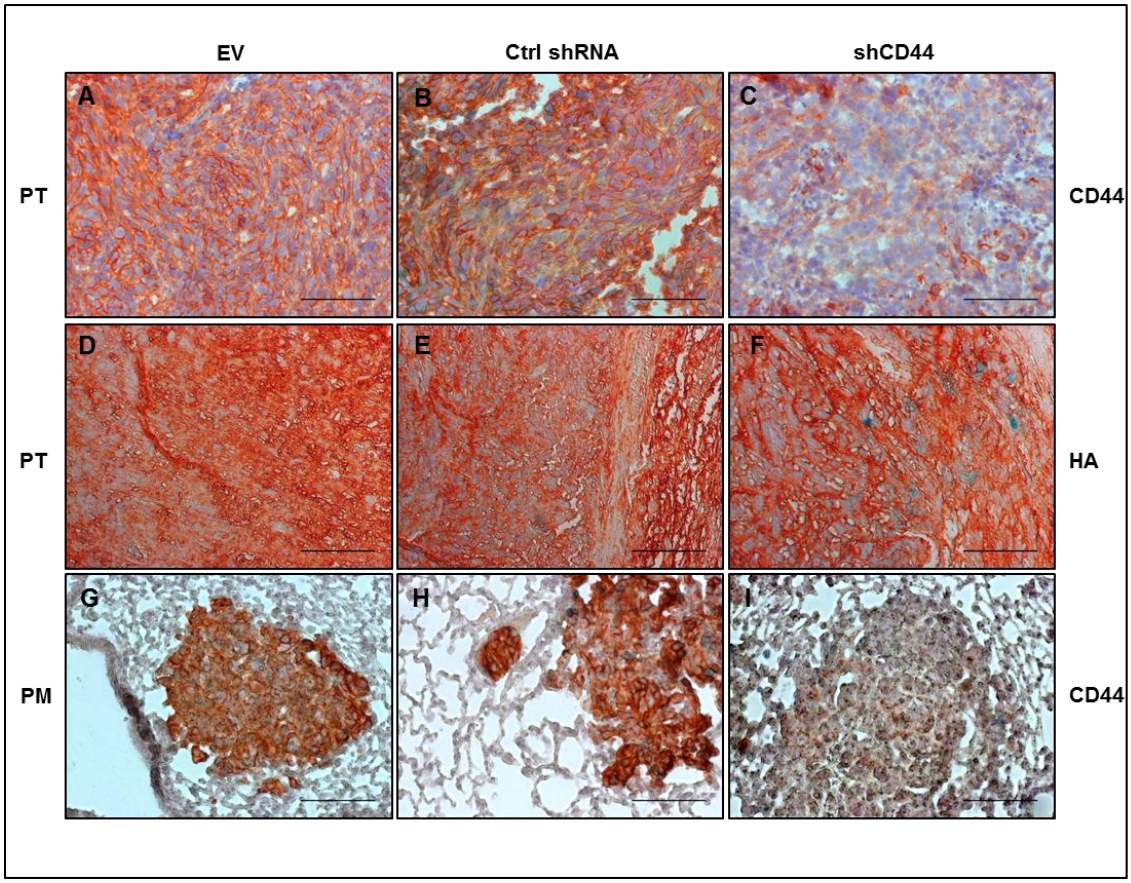


Figure 4.

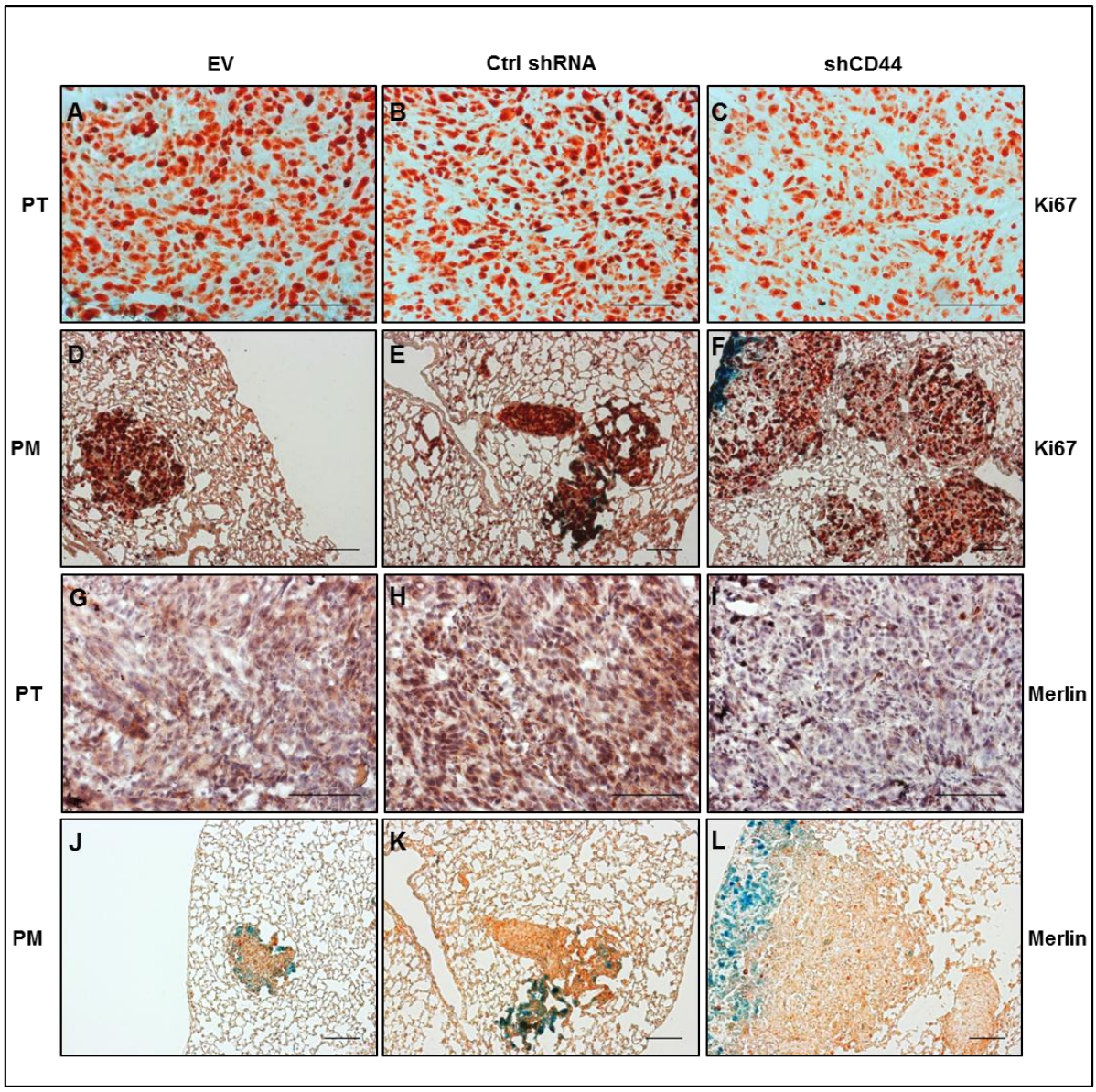

EV
Ctrl shRNA
shCD44
A
B
C
PT
Ki67
D
E
F
PM
Ki67
G
H
I
PT
Merlin
J
K
L
PM
Merlin

Figure 5.

4.3 Additional studies:

Effects of CD44v6 overexpression on tumorigenic and metastatic properties of human SaOS-2 osteosarcoma cells in vitro and in vivo

4.3.1 Results

The first major finding that showed a role for CD44 in metastatic spread was the discovery that the CD44 isoform containing the sequence encoded by the v6 exon confers metastatic potential to non-metastasizing rat pancreatic carcinoma cells (Günthert et al., 1991). In the following decades, numerous reports linked CD44v6 expression to poor prognosis of patients suffering from various cancer types including colorectal, cervical, gastric and breast cancer (Orian-Rousseau, 2010). Interestingly, Kuryu et al. found that overexpression of CD44v6 in tumor specimens correlated with a poor prognosis in osteosarcoma (OS) patients (Kuryu et al., 1999).

Therefore, in order to determine the potential effects of CD44v6 expression on the tumorigenicity and the metastatic behavior of osteosarcoma cells, we overexpressed by retroviral gene transfer a C-terminally V5/His6 tagged CD44v6 isoform in low-metastatic SaOS-2/*LacZ* cells (SaOS-2 CD44v6 cells), with no endogenous CD44v6. Control cells SaOS-2/*LacZ* were transduced with the pQCIXH empty vector (EV) to reveal SaOS-2 EV. Stable overexpression of CD44v6 protein was confirmed by Western blot analysis of cell extracts and immunocytochemistry (Figure Z1). The transductants were further characterized *in vitro* in functional assays as well as *in vivo* for their ability to form primary tumors and pulmonary metastases.

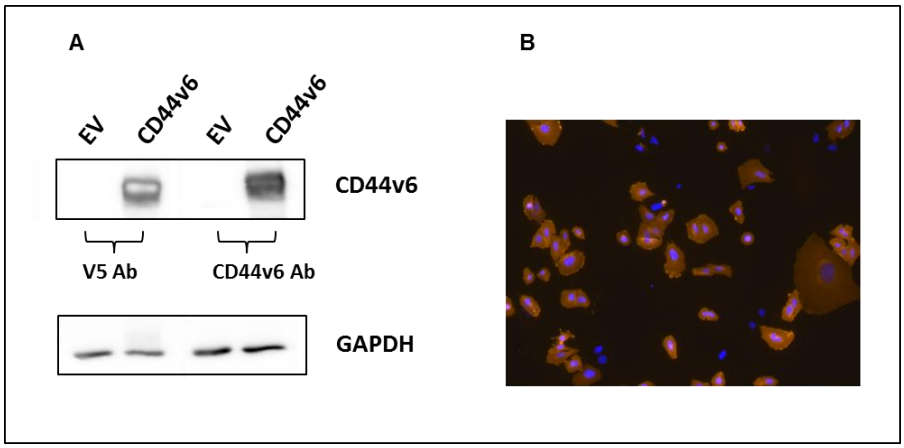


Figure Z1. **CD44v6 overexpression in low-metastatic human SaOS-2 OS cells.** (A) Overexpression of CD44v6-V5/His6 achieved through retroviral gene transfer was confirmed by Western blot analysis using both V5 and CD44v6 antibodies. GAPDH was used as a loading control. (B) Immunocytochemistry performed with anti-V5 antibody revealed CD44v6 overexpression in approximately 80% of cells.

CD44v6 overexpression does not alter the adhesion to immobilized hyaluronan, but it increases the migration capacity and inhibits proliferation of SaOS-2 cells *in vitro*

First, we tested the stable transductants for short term adhesion to hyaluronan (HA), the major ligand for CD44. SaOS-2 CD44v6 cell showed a2.3- fold increased adhesion compared to the EV control cells ($p < 0.25$; Fig Z2A). We further examined the potential effect of CD44v6 overexpression on the migratory properties of SaOS-2 cells in a transwell migration assay. CD44v6 overexpression resulted in a significant 8-fold increase in the migration rates when compared to EV cells ($p < 0.05$; Fig Z2B). Next, we explored whether CD44v6 overexpression has any impact on the proliferation of SaOS-2 cells *in vitro*. Proliferation of SaOS-2 CD44v6 cells was significantly ($p < 0.05$) decreased when compared to that of SaOS-2 EV cells, and the calculated doubling times were 38.2 ± 1.6h for SaOS-2 EV and 43.7 ± 1.6h for SaOS-2 CD44v6 cells (Fig Z2C).

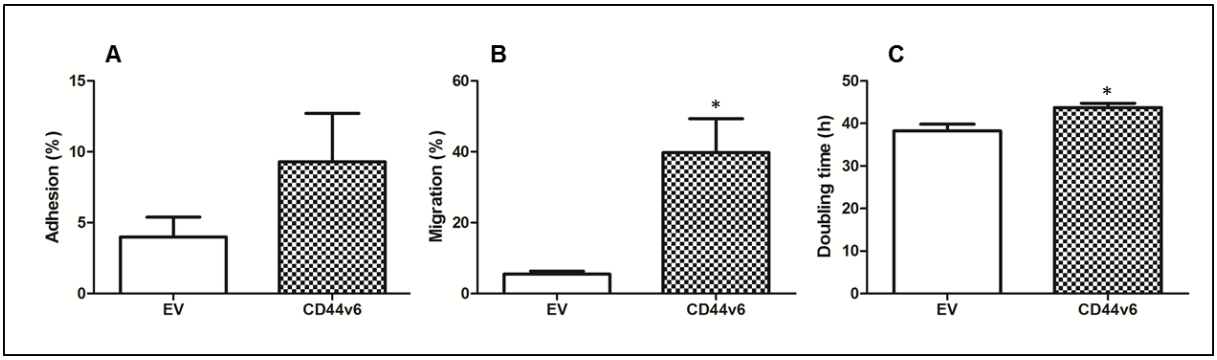


Figure Z2. Overexpression of CD44v6 in SaOS-2 cells promotes migration and inhibits proliferation, but does not alter adhesion to HA. (A) Short-term (30 min) adhesion of SaOS-2 EV and SaOS-2 CD44v6 cells to immobilized HA. Migration rates assessed in a transwell migration assay (B) and (C) proliferation of EV and CD44v6 transduced SaOS-2 cells. Values are expressed as means ± SEM of at least three independent experiments; *, $p < 0.05$.

CD44v6 overexpression in osteoblastic SaOS-2 cells does not have a significant impact on intratibial primary tumor growth and the number of pulmonary macrometastases, but significantly decreases the number of micrometastases

After the characterization of CD44v6 overexpressing SaOS-2 cells *in vitro*, we investigated the tumorigenic and metastatic properties of SaOS-2 CD44v6 cells *in vivo*. 5 x 10^5 of SaOS-2 EV or SaOS-2 CD44v6 cells were intratibially injected into immunocompromised SCID/CB17 mice. Primary tumor growth and resulting osteoblastic lesions were monitored by X-ray (Figure Z3B). In the 12^{th} week after tumor cell injection, mice became moribund and were consequently sacrificed. Osteoblastic lesions in the tumor-injected legs were comparable in the two groups of animals. Tumors derived from SaOS-2 CD44v6 cells tended to grow more slowly than those developing from SaOS-2 EV cells ($p < 0.08$; Fig Z4A). Mean calculated final volumes of SaOS-2 CD44v6 and SaOS-2 EV xenografts were 72 ± 19 mm^3 and 143 ± 28 mm^3, respectively.

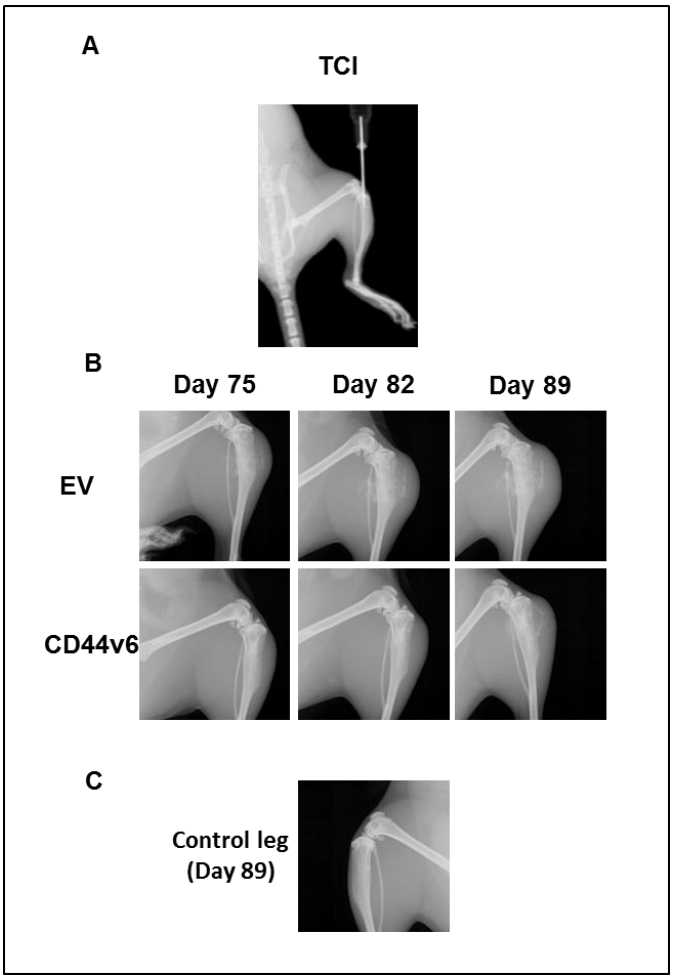


Figure Z3. X-ray monitoring of primary tumor development upon intratibial injection of SaOS-2 EV and SaOS-2 CD44v6 cells into SCID mice. (A) Representative X-ray control image confirming the correct position of the Hamilton syringe during intratibial tumor cell injection (TCI). (B) Representative X-ray images of tumor-bearing legs taken on indicated day after injection of SaOS-2 EV cells (11 mice) or of SaOS-2 CD44v6 cells (7 mice). (C) Representative X-ray image of a non-injected control leg.

As described previously, the cells used were transduced with a *LacZ* gene which allowed *ex vivo* X-Gal staining of whole lung mounts and thereby tracking of tumor cells in mouse tissue and quantification of pulmonary metastatic nodules. Mean number of lung macrometastases did not significantly differ between the mice bearing SaOS-2 CD44v6 cell-derived xenografts and mice with SaOS-2 EV cell-derived tumors ($p > 0.5$; Figure Z4B). Mice with CD44v6 overexpressing tumors, on the other hand, showed a 2.2-fold higher mean number of lung micrometastases than mice with SaOS-2 EV cell derived tumors ($p < 0.05$; Figure Z4C).

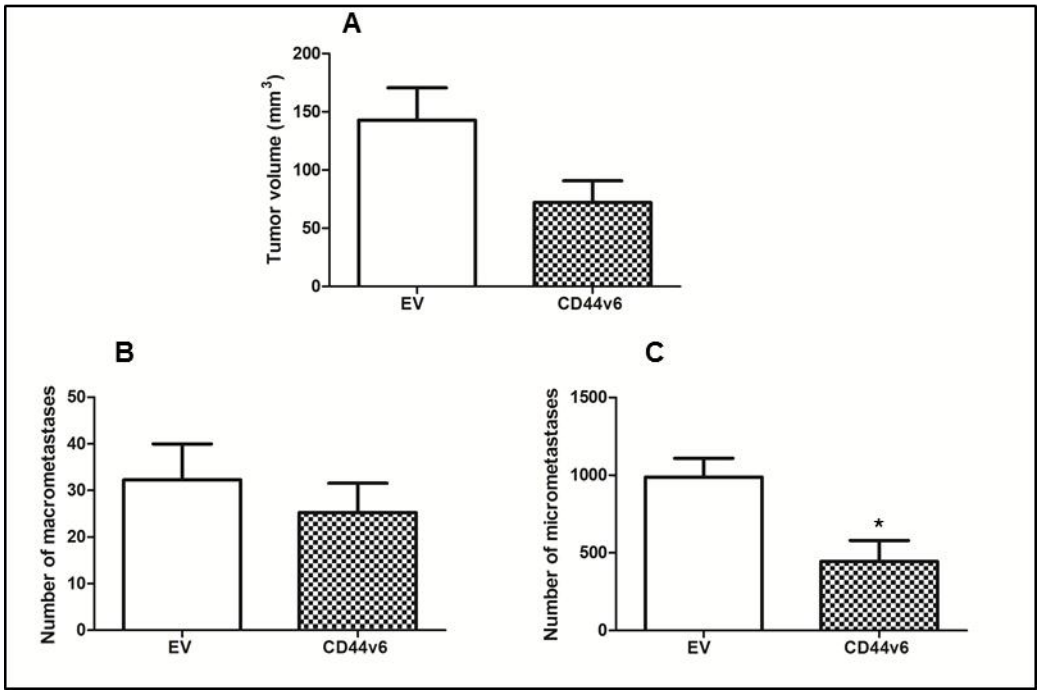


Figure Z4. Primary tumor growth and formation of pulmonary metastases upon intratibial injection of SaOS-2 EV and SaOS-2 CD44v6 cells into SCID mice. (A) Mean (± SEM) primary tumor volume of mice bearing SaOS-2 EV cell-derived (n=11) or SaOS-2 CD44v6 cell-derived (n=7) tumors 12 weeks after tumor cell injection. Quantification of pulmonary macrometastases (B) and micrometastases (C) on whole lung mounts prepared after sacrifice of the mice in week 12 after tumor cells injection. Values are presented as mean ± SEM; *, $p < 0.05$

4.3.2 Materials and Methods

Generation of CD44v6 expression construct and retroviral infection

A cDNA fragment encoding the v6 exon of CD44 was amplified by RT/PCR from total RNA isolated from human 143-B cells (ATCC; CRL-8303) using the following primers:

- 5'-ACTATTGTTAACCGTGATGGCACCC-3'
- 5'-ATTCTCGAGTCACCCCAATCTTCATGT-3'.

The PCR product was cloned into the pGEM®-T Easy Vector (Promega, Madison, WI) and then inserted at the proper position of the nucleotide sequence encoding CD44s with C-terminal V5 and His6 tags, which had earlier been cloned into the retroviral expression vector pQCIXH. This gave rise to pQCIXH CD44v6-V5/His6. The sequence was verified by sequencing of both strands. The retroviral particles containing pQCIXH EV (empty vector) and pQCIXH CD44v6-V5/His were generated in HEK 293T (ATCC; CRL-11268) cells and subsequently used to infect SaOS-2/*LacZ* cells as described (Arlt et al., 2011). Selection with hygromycin (400 µg/ml) revealed SaOS-2 EV and SaOS CD44v6 stable cell lines. Apecific anti-CD44v6 antibody used for detection of CD44v6 in WB at the dilution of 1:1000 was purchased from Bender MedSystems.

Western blot, immunocytochemistry, adhesion assay, transwell migration assay, proliferation assay and the animal experiments were all performed as described in detail in the *Material and Methods* section in Manusript 1.

4.3.3 Discussion

Only two immunohistochemichal studies revealed correlation of CD44 variant expression in OS tumor samples and poor prognosis for the patients, however one study claimed CD44v5 to be important, whereas the other found CD44v6 to be relevant for patients outcome (Kim et al., 2002; Kuryu et al., 1999). Using primers that span the variant exons, we performed expression analysis of CD44 isoform expressed in 8 established human OS cell lines and found CD44 standard isoform to be predominantly expressed. CD44v6 isoform expression was observed as a minor fraction in 5 out of 8 cell lines (data not shown). Therefore, we aimed at investigating the *in vitro* and *in vivo* effects of CD44v6 overexpression in SaOS-2 on their tumorigenic and metastatic behavior. The SaOS-2 cells were chosen for manipulation experiments because they are negative for CD44v6 protein expression, and have almost undetectable CD44s expression. Stable CD44v6 overexpressing transductants showed only a tendency of higher adhesion to HA *in vitro* when compared to that of control cells. As described in the thesis introduction, the HA-binding site is located in the standard part, but the insertion of variant exons and glycosylation state that is cell-type specific can significantly modify the ability to bind HA. Similar to our results, a CD44 variant containing v6 exon encoded sequence when overexpressed in human Burkitt lymphoma Namalwa cells only slightly increased adhesion to HA (Bartolazzi et al., 1995). On the contrary to the adhesion, CD44v6 overexpression notably promoted the migration of SaOS-2 cells *in vitro*. Although the mechanism of CD44v6 regulated migration in OS needs to be clarified, the likely explanation would be analogous to some human carcinoma cell lines where the CD44v6 isoform forms a complex with c-Met/HGF that subsequently promotes cell motility (Orian-Rousseau et al., 2002). CD44v6 overexpression decreased the *in vitro* proliferation rate of SaOS-2 cells. To summarize the *in vitro* effects of CD44v6 overexpression, it strongly enhanced migration, an important indicator of metastatic activity, however, it reduced the growth rate, an indicator of tumorigenicity. With the use of the

xenograft OS mouse model, we found that CD44v6 overexpressing SaOS-2 cells showed only a tendency of forming smaller tumors compared to control cells upon intratibial injection and this finding is in line with our *in vitro* observations of slower tumor cell growth. On the other hand, outgrowth of pulmonary metastases did not significantly differ in mice of the experimental and control groups, which was reflected in the similar number of formed macrometastases. However, the number of micrometastases in the lungs of mice bearing SaOS-2 CD44v6 cell-derived tumors was significantly reduced compared to mice with SaOS-2 EV-derived tumors. As the number of SaOS-2 CD44v6-originating macrometastases is comparable and the number of micrometastases is decreased when compared to that formed by SaOS-2 EV cells, we suspect that CD44v6-overexpressing cells disseminate within the lung tissue, eventually giving rise to nodules larger than 0.1mm in diameter. Additional experiments are required to determine which lung tissue factors interplay with CD44v6-overexpressing tumor cells leading to their elevated dissemination and outgrowth in the lungs.

In conclusion, CD44v6, distinct from CD44s, does not appear to play a relevant role in regulating OS progression and metastasis in this particular orthotopic xenograft OS mouse model. The findings presented here underline the importance of CD44s, but not variant CD44 isoforms, for OS progression, as discussed in Manuscript 1.

5 Conclusion and Outlook

Pulmonary metastases are the major cause of death in OS patients and despite the clinical advances and the use of multi-modality treatment patients suffering from metastatic disease continue to have a very poor prognosis. The development of successful disease management strategies that eradicate metastases requires detailed understanding of molecular mechanisms and knowledge on key regulators of increased metastatic spread and/or chemoresistance. Acquiring profound knowledge of fundamental biology of OS and identification of novel OS molecular markers will aid the design of targeted effective treatment of this devastating disease.

In the thesis presented here, the prognostic value of CD44 expression for OS patients' outcome and the biological relevance of CD44/HA interactions for OS malignancy in orthotopic xenograft OS mouse models were investigated.

Our tissue microarray analysis of OS tumor specimens identified CD44 as an additional indicator for poor patient's outcome together with the lack of response to chemotherapy and the presence of metastases, that are up to date the most powerful and reliable predictors for a poor prognosis for OS patients. We consider CD44 as a molecular marker that can be used for stratification of patients into different-risk groups. This sets a framework for personalized treatment, which allows tailored therapy for individual patients according to the molecular profile of their tumors. The ultimate goal is to enhance treatment efficacy while minimizing the adverse side effects of anticancer drugs, which is especially important for the treatment of rapidly growing pediatric patients. In our cohort of patients that poorly respond to neoadjuvant chemotherapy and of patients with metastases, those with CD44 expression by the tumor represent a high-risk group and may therefore be considered as candidates for novel therapeutic strategies.

At the same time, our results in experimental models of OS highlight CD44/HA interaction as a promising target for therapeutic intervention in OS. Making use of a xenograft orthotopic OS mouse model, that closely reproduces the human disease, we demonstrate for the first time that CD44s, but not CD44v6, has a tumor- and metastatis-promoting activity upon overexpression in a human non-transformed osteoblastic SaOS-2 cell line. The malignancy enhancing effect of CD44s was HA-dependent. In addition to enhancing the tumorigenic and metastatic behavior of SaOS-2 cells, CD44 was found to be associated with chemoresistance to cisplatin *in vitro*. Future studies should be focused on addressing the possible signaling pathways involved and on exploring the potential CD44 linked chemoresistance *in vivo*.

Conversely, when we explored the contribution of CD44 to the OS malignant phenotype in another xenograft model of OS, we found that CD44 acts as a metastasis suppressor in this particular experimental setup. Namely, CD44 silencing in the highly metastatic human 143-B cell line resulted in increased metastatic potential probably due to reduced adhesion at the primary tumor site and loss of expression of the metastasis suppressor merlin *in vivo*. 143-B cell line is a Ki-Ras transformed cell line, and, although at this point we cannot exclude the possibility of a cell-type specific effect of CD44 down-regulation, it is reasonable to suggest that CD44 may act as a negative regulator of metastasis in a subset of OS where Ras signaling is involved.

Various approaches have been described in the literature to interfere with CD44/HA interactions *in vitro* and *in vivo* in different murine tumor models, including a few studies in experimental OS. Along these lines, exchange of endogenous multivalent HA with small monovalent HA oligomers (Hosono et al., 2007), the use of antisense inhibition of HA synthase 2 (Nishida et al., 2005) or treatment with an inhibitor of HA synthesis 4-methylumbelliferon (Arai et al., 2011) have been reported. However, a functional inhibition

of CD44 by displacement of endogenous HA with the use of soluble HA-binding CD44 proteins, that we are investigating in addition to our CD44 silencing experiments, as well as inhibition of CD44/HA interaction with blocking antibodies (e.g. Hermes1), may in the future clarify the relevance of CD44 in different mouse models of OS.

Taken together, we can conclude that CD44 may have a dual role in regulating OS progression and metastasis depending on the cellular background or the experimental models used, as shown in breast cancer (Louderbough and Schroeder, 2011). However, in the context of osteoblastic OS, the most common type of OS, our study demonstrated that CD44s promotes OS growth and dissemination and its interaction with HA may serve as a potential therapeutic target. In addition to targeting CD44/HA interactions directly, these interactions can also be utilized for the delivery of anticancer drugs to CD44 expressing tumor cells. For example, drug-loaded liposomes or nanoparticles decorated with HA or anti-CD44 antibody showed efficacy in animal tumor models (Platt and Szoka, 2008). Initial trials with anti-CD44v6 antibodies coupled to radioisotopes or cytotoxic drugs in head and neck squamous cell carcinoma and breast cancer patients showed some clinical success, although complications with various toxicities occurred (Orian-Rousseau, 2010). As CD44s is an abundantly expressed molecule that plays central roles in numerous physiological processes, there is concern regarding selective targeting of cancer cells. Nevertheless, it appears that malignant tumors have higher sensitivity to disruption of CD44/HA interaction than normal physiological processes (Toole, 2009). In order to avoid adverse side effects, the most promising strategy would be to use bispecific antibodies, targeting two different antigens on a cancer cell, as it was shown for CD44 and a leukemia idiotype (Avin et al., 2004).

Because of the high degree of heterogeneity of OS tumor cells, future investigations in additional OS cell lines, ideally primary human OS cells, are likely to give

informative results finally leading to detailed delineation of the relevance of CD44/HA interaction for different subtypes of OS.

In conclusion, results presented in this thesis clearly demonstrate that, at least in experimental osteoblastic OS, CD44s/HA interaction plays a malignancy promoting role and that these molecules are promising targets for successful treatment of this highly aggressive cancer type.

6 References

Ahrens T, Sleeman JP, Schempp CM, Howells N, Hofmann M, Ponta H, Herrlich P, Simon JC (2001) Soluble CD44 inhibits melanoma tumor growth by blocking cell surface CD44 binding to hyaluronic acid. Oncogene 20:3399-3408

Aisen AM, Martel W, Braunstein EM, McMillin KI, Phillips WA, Kling TF (1986) MRI and CT evaluation of primary bone and soft-tissue tumors. AJR Am J Roentgenol 146:749-756

Angelo M. De Marzo CBJSJIEGJM (1998) CD44 and CD44v6 downregulation in clinical prostatic carcinoma: Relation to Gleason grade and cytoarchitecture. vol 34, Prostate, pp 162-168

Anttila MA, Tammi RH, Tammi MI, Syrjanen KJ, Saarikoski SV, Kosma VM (2000) High levels of stromal hyaluronan predict poor disease outcome in epithelial ovarian cancer. Cancer Res 60:150-155

Arai E, Nishida Y, Wasa J, Urakawa H, Zhuo L, Kimata K, Kozawa E, Futamura N, Ishiguro N (2011) Inhibition of hyaluronan retention by 4-methylumbelliferone suppresses osteosarcoma cells in vitro and lung metastasis in vivo. Br J Cancer

Arlt MJ, Banke IJ, Walters DK, Puskas GJ, Steinmann P, Muff R, Born W, Fuchs B (2011) LacZ transgene expression in the subcutaneous Dunn/LM8 osteosarcoma mouse model allows for the identification of micrometastasis. J Orthop Res

Aruffo A, Stamenkovic I, Melnick M, Underhill CB, Seed B (1990) CD44 is the principal cell surface receptor for hyaluronate. Cell 61:1303-1313

Aung L, Gorlick RG, Shi W, Thaler H, Shorter NA, Healey JH, Huvos AG, Meyers PA (2002) Second malignant neoplasms in long-term survivors of osteosarcoma: Memorial Sloan-Kettering Cancer Center Experience. Cancer 95:1728-1734

Auvinen P, Tammi R, Parkkinen J, Tammi M, Agren U, Johansson R, Hirvikoski P, Eskelinen M, Kosma VM (2000) Hyaluronan in peritumoral stroma and malignant cells associates with breast cancer spreading and predicts survival. Am J Pathol 156:529-536

Avin E, Haimovich J, Hollander N (2004) Anti-idiotype x anti-CD44 bispecific antibodies inhibit invasion of lymphoid organs by B cell lymphoma. J Immunol 173:4736-4743

Bacac M, Stamenkovic I (2008) Metastatic cancer cell. Annu Rev Pathol 3:221-247

Bacci G, Ferrari S, Longhi A, Picci P, Mercuri M, Alvegard TA, Saeter G, Donati D, Manfrini M, Lari S, Briccoli A, Forni C (2002) High dose ifosfamide in combination with high dose methotrexate, adriamycin and cisplatin in the neoadjuvant treatment of extremity osteosarcoma: preliminary results of an Italian Sarcoma Group/Scandinavian Sarcoma Group pilot study. J Chemother 14:198-206

Balzer EM, Konstantopoulos K (2011) Intercellular adhesion: mechanisms for growth and metastasis of epithelial cancers. Wiley Interdiscip Rev Syst Biol Med

Bartolazzi A, Jackson D, Bennett K, Aruffo A, Dickinson R, Shields J, Whittle N, Stamenkovic I (1995) Regulation of growth and dissemination of a human lymphoma by CD44 splice variants. J Cell Sci 108 (Pt 4):1723-1733

Bennett KL, Jackson DG, Simon JC, Tanczos E, Peach R, Modrell B, Stamenkovic I, Plowman G, Aruffo A (1995) CD44 isoforms containing exon V3 are responsible for the presentation of heparin-binding growth factor. J Cell Biol 128:687-698

Bielack SS, Carrle D, Hardes J, Schuck A, Paulussen M (2008) Bone tumors in adolescents and young adults. Curr Treat Options Oncol 9:67-80

Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, Kotz R, Salzer-Kuntschik M, Werner M, Winkelmann W, Zoubek A, Jurgens H, Winkler K (2002) Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol 20:776-790

Boldrini E, Peres SV, Morini S, de Camargo B (2010) Immunoexpression of Ezrin and CD44 in patients with osteosarcoma. J Pediatr Hematol Oncol 32:e213-217

Bono P, Rubin K, Higgins JM, Hynes RO (2001) Layilin, a novel integral membrane protein, is a hyaluronan receptor. Mol Biol Cell 12:891-900

Bourguignon LY, Zhu D, Zhu H (1998) CD44 isoform-cytoskeleton interaction in oncogenic signaling and tumor progression. Front Biosci 3:d637-649

Bourguignon LY, Zhu H, Chu A, Iida N, Zhang L, Hung MC (1997) Interaction between the adhesion receptor, CD44, and the oncogene product, p185HER2, promotes human ovarian tumor cell activation. J Biol Chem 272:27913-27918

Bretscher A, Edwards K, Fehon RG (2002) ERM proteins and merlin: integrators at the cell cortex. Nat Rev Mol Cell Biol 3:586-599

Bridge JA, Nelson M, McComb E, McGuire MH, Rosenthal H, Vergara G, Maale GE, Spanier S, Neff JR (1997) Cytogenetic findings in 73 osteosarcoma specimens and a review of the literature. Cancer Genetics and Cytogenetics 95:74-87

Campanacci M, Bacci G, Bertoni F, Picci P, Minutillo A, Franceschi C (1981) The treatment of osteosarcoma of the extremities: twenty year's experience at the Istituto Ortopedico Rizzoli. Cancer 48:1569-1581

Cannistra SA, Kansas GS, Niloff J, DeFranzo B, Kim Y, Ottensmeier C (1993) Binding of Ovarian Cancer Cells to Peritoneal Mesothelium in Vitro Is Partly Mediated by CD44H. Cancer Research 53:3830-3838

Carrle D, Bielack S (2009) Osteosarcoma lung metastases detection and principles of multimodal therapy. Cancer Treat Res 152:165-184

Chambers AF, Groom AC, MacDonald IC (2002) Metastasis: Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer 2:563-572

Chiang AC, Massague J (2008) Molecular basis of metastasis. N Engl J Med 359:2814-2823
Clark JC, Dass CR, Choong PF (2008) A review of clinical and molecular prognostic factors in osteosarcoma. J Cancer Res Clin Oncol 134:281-297

Colombatti A, Hughes EN, Taylor BA, August JT (1982) Gene for a major cell surface glycoprotein of mouse macrophages and other phagocytic cells is on chromosome 2. Proc Natl Acad Sci U S A 79:1926-1929

Croker AK, Allan AL (2008) Cancer stem cells: implications for the progression and treatment of metastatic disease. J Cell Mol Med 12:374-390

de Kraker J, Voute PA (1989) Experience with ifosfamide in paediatric tumours. Cancer chemotherapy and pharmacology 24 Suppl 1:S28-29

Delpech B, Chevallier B, Reinhardt N, Julien JP, Duval C, Maingonnat C, Bastit P, Asselain B (1990) Serum hyaluronan (hyaluronic acid) in breast cancer patients. Int J Cancer 46:388-390

Delpech B, Laquerriere A, Maingonnat C, Bertrand P, Freger P (2002) Hyaluronidase is more elevated in human brain metastases than in primary brain tumours. Anticancer Res 22:2423-2427

Enneking WF (1979) Advances and treatment of primary bone tumors. The Journal of the Florida Medical Association 66:28-30

Enneking WF, Spanier SS, Goodman MA (1980) A system for the surgical staging of musculoskeletal sarcoma. Clin Orthop Relat Res:106-120

Ewing J (1928) Neoplastic Diseases: A Treatise on Tumours. By James Ewing, A.M., M.D., Sc.D., Professor of Pathology at Cornell University Medical College, N.Y.; Pathologist to the Memorial Hospital. Third edition. Royal 8vo. Pp. 1127, with 546 illustrations. 1928. Philadelphia and London: W. B. Saunders Co. Ltd. 63s. net. British Journal of Surgery 16:174-175

Fidler IJ (2003) The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. Nat Rev Cancer 3:453-458

Fidler IJ, Kripke ML (1977) Metastasis results from preexisting variant cells within a malignant tumor. Science 197:893-895

Franzmann EJ, Schroeder GL, Goodwin WJ, Weed DT, Fisher P, Lokeshwar VB (2003) Expression of tumor markers hyaluronic acid and hyaluronidase (HYAL1) in head and neck tumors. Int J Cancer 106:438-445

Frost GI, Mohapatra G, Wong TM, Csoka AB, Gray JW, Stern R (2000) HYAL1LUCA-1, a candidate tumor suppressor gene on chromosome 3p21.3, is inactivated in head and neck squamous cell carcinomas by aberrant splicing of pre-mRNA. Oncogene 19:870-877

Fuchs B, Pritchard DJ (2002) Etiology of osteosarcoma. Clin Orthop Relat Res:40-52

Fujimoto T, Kawashima H, Tanaka T, Hirose M, Toyama-Sorimachi N, Matsuzawa Y, Miyasaka M (2001) CD44 binds a chondroitin sulfate proteoglycan, aggrecan. Int Immunol 13:359-366

Gao AC, Lou W, Dong J-T, Isaacs JT (1997) CD44 Is a Metastasis Suppressor Gene for Prostatic Cancer Located on Human Chromosome 11p13. vol 57, pp 846-849

Glasser DB, Lane JM, Huvos AG, Marcove RC, Rosen G (1992) Survival, prognosis, and therapeutic response in osteogenic sarcoma. The Memorial Hospital experience. Cancer 69:698-708

Goldstein LA, Butcher EC (1990) Identification of mRNA that encodes an alternative form of H-CAM(CD44) in lymphoid and nonlymphoid tissues. Immunogenetics 32:389-397

Goodfellow PN, Banting G, Wiles MV, Tunnacliffe A, Parkar M, Solomon E, Dalchau R, Fabre JW (1982) The gene, MIC4, which controls expression of the antigen defined by monoclonal antibody F10.44.2, is on human chromosome 11. Eur J Immunol 12:659-663

Günthert U, Hofmann M, Rudy W, Reber S, Zöller M, Haumann I, Matzku S, Wenzel A, Ponta H, Herrlich P (1991) A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. Cell 65:13-24

Gupta GP, Nguyen DX, Chiang AC, Bos PD, Kim JY, Nadal C, Gomis RR, Manova-Todorova K, Massague J (2007) Mediators of vascular remodelling co-opted for sequential steps in lung metastasis. Nature 446:765-770

Hart IR, Fidler IJ (1980) Role of organ selectivity in the determination of metastatic patterns of B16 melanoma. Cancer Res 40:2281-2287

Hautmann SH, Lokeshwar VB, Schroeder GL, Civantos F, Duncan RC, Gnann R, Friedrich MG, Soloway MS (2001) Elevated tissue expression of hyaluronic acid and hyaluronidase validates the HA-HAase urine test for bladder cancer. J Urol 165:2068-2074

Heider K-H, Dämmrich J, Skroch-Angel P, Müller-Hermelink H-K, Vollmers HP, Herrlich P, Ponta H (1993) Differential Expression of CD44 Splice Variants in Intestinal- and Diffuse-Type Human Gastric Carcinomas and Normal Gastric Mucosa. Cancer Research 53:4197-4203

Helman LJ, Meltzer P (2003) Mechanisms of sarcoma development. Nat Rev Cancer 3:685-694
Herrlich P, Sleeman J, Wainwright D, Konig H, Sherman L, Hilberg F, Ponta H (1998) How tumor cells make use of CD44. Cell Adhes Commun 6:141-147

Hosono K, Nishida Y, Knudson W, Knudson CB, Naruse T, Suzuki Y, Ishiguro N (2007) Hyaluronan oligosaccharides inhibit tumorigenicity of osteosarcoma cell lines MG-63 and LM-8 in vitro and in vivo via perturbation of hyaluronan-rich pericellular matrix of the cells. Am J Pathol 171:274-286

Hu G, Chong RA, Yang Q, Wei Y, Blanco MA, Li F, Reiss M, Au JLS, Haffty BG, Kang Y (2009) MTDH Activation by 8q22 Genomic Gain Promotes Chemoresistance and Metastasis of Poor-Prognosis Breast Cancer. Cancer Cell 15:9-20

Hudson DL, Sleeman J, Watt FM (1995) CD44 is the major peanut lectin-binding glycoprotein of human epidermal keratinocytes and plays a role in intercellular adhesion. J Cell Sci 108 (Pt 5):1959-1970

Huvos AG (1981) Bone Tumors: Diagnosis, Treatment and Prognosis. Journal of Pediatric Hematology/Oncology 3:221

Itano N, Sawai T, Miyaishi O, Kimata K (1999) Relationship between hyaluronan production and metastatic potential of mouse mammary carcinoma cells. Cancer Res 59:2499-2504

Jacobson A, Rahmanian M, Rubin K, Heldin P (2002) Expression of hyaluronan synthase 2 or hyaluronidase 1 differentially affect the growth rate of transplantable colon carcinoma cell tumors. Int J Cancer 102:212-219

Joyce JA, Pollard JW (2009) Microenvironmental regulation of metastasis. Nat Rev Cancer 9:239-252
Kalomiris EL, Bourguignon LY (1989) Lymphoma protein kinase C is associated with the transmembrane glycoprotein, GP85, and may function in GP85-ankyrin binding. J Biol Chem 264:8113-8119

Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordon-Cardo C, Guise TA, Massague J (2003) A multigenic program mediating breast cancer metastasis to bone. Cancer Cell 3:537-549

Kaplan RN, Rafii S, Lyden D (2006) Preparing the "soil": the premetastatic niche. Cancer Res 66:11089-11093

Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, MacDonald DD, Jin DK, Shido K, Kerns SA, Zhu Z, Hicklin D, Wu Y, Port JL, Altorki N, Port ER, Ruggero D, Shmelkov SV, Jensen KK, Rafii S, Lyden D (2005) VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature 438:820-827

Karjalainen JM, Tammi RH, Tammi MI, Eskelinen MJ, Agren UM, Parkkinen JJ, Alhava EM, Kosma VM (2000) Reduced level of CD44 and hyaluronan associated with unfavorable prognosis in clinical stage I cutaneous melanoma. Am J Pathol 157:957-965

Katagiri YU, Sleeman J, Fujii H, Herrlich P, Hotta H, Tanaka K, Chikuma S, Yagita H, Okumura K, Murakami M, Saiki I, Chambers AF, Uede T (1999) CD44 variants but not CD44s cooperate with beta1-containing integrins to permit cells to bind to osteopontin independently of arginine-glycine-aspartic acid, thereby stimulating cell motility and chemotaxis. Cancer Res 59:219-226

Kawashima H, Hirose M, Hirose J, Nagakubo D, Plaas AH, Miyasaka M (2000) Binding of a large chondroitin sulfate/dermatan sulfate proteoglycan, versican, to L-selectin, P-selectin, and CD44. J Biol Chem 275:35448-35456

Kim HS, Park YB, Oh JH, Jeong J, Kim CJ, Lee SH (2002) Expression of CD44 isoforms correlates with the metastatic potential of osteosarcoma. Clin Orthop Relat Res:184-190

Kosaki R, Watanabe K, Yamaguchi Y (1999) Overproduction of hyaluronan by expression of the hyaluronan synthase Has2 enhances anchorage-independent growth and tumorigenicity. Cancer Res 59:1141-1145

Kuryu M, Ozaki T, Nishida K, Shibahara M, Kawai A, Inoue H (1999) Expression of CD44 variants in osteosarcoma. J Cancer Res Clin Oncol 125:646-652

Langley RR, Fidler IJ (2007) Tumor cell-organ microenvironment interactions in the pathogenesis of cancer metastasis. Endocr Rev 28:297-321

Legg JW, Lewis CA, Parsons M, Ng T, Isacke CM (2002) A novel PKC-regulated mechanism controls CD44 ezrin association and directional cell motility. Nat Cell Biol 4:399-407

Lim S, Young A, Paner G, Amin M (2008) Prognostic role of CD44 cell adhesion molecule expression in primary and metastatic renal cell carcinoma: a clinicopathologic study of 125 cases. Virchows Archiv 452:49-55

Lipponen P, Aaltomaa S, Tammi R, Tammi M, Agren U, Kosma VM (2001) High stromal hyaluronan level is associated with poor differentiation and metastasis in prostate cancer. Eur J Cancer 37:849-856

Lipshultz SE, Lipsitz SR, Mone SM, Goorin AM, Sallan SE, Sanders SP, Orav EJ, Gelber RD, Colan SD (1995) Female Sex and Higher Drug Dose as Risk Factors for Late Cardiotoxic Effects of Doxorubicin Therapy for Childhood Cancer. New England Journal of Medicine 332:1738-1744

Liu N, Gao F, Han Z, Xu X, Underhill CB, Zhang L (2001) Hyaluronan synthase 3 overexpression promotes the growth of TSU prostate cancer cells. Cancer Res 61:5207-5214

Lokeshwar VB, Fregien N, Bourguignon LY (1994) Ankyrin-binding domain of CD44(GP85) is required for the expression of hyaluronic acid-mediated adhesion function. J Cell Biol 126:1099-1109

Lokeshwar VB, Schroeder GL, Selzer MG, Hautmann SH, Posey JT, Duncan RC, Watson R, Rose L, Markowitz S, Soloway MS (2002) Bladder tumor markers for monitoring recurrence and screening comparison of hyaluronic acid-hyaluronidase and BTA-Stat tests. Cancer 95:61-72

Longhi A, Errani C, De Paolis M, Mercuri M, Bacci G (2006) Primary bone osteosarcoma in the pediatric age: state of the art. Cancer Treat Rev 32:423-436

Louderbough JM, Schroeder JA (2011) Understanding the dual nature of CD44 in breast cancer progression. Mol Cancer Res 9:1573-1586

Luzzi KJ, MacDonald IC, Schmidt EE, Kerkvliet N, Morris VL, Chambers AF, Groom AC (1998) Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. Am J Pathol 153:865-873

Marhaba R, Zoller M (2004) CD44 in cancer progression: adhesion, migration and growth regulation. J Mol Histol 35:211-231

Marina N, Gebhardt M, Teot L, Gorlick R (2004) Biology and Therapeutic Advances for Pediatric Osteosarcoma. Oncologist 9:422-441

Meyer D, Birchmeier C (1995) Multiple essential functions of neuregulin in development. Nature 378:386-390

Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, Viale A, Olshen AB, Gerald WL, Massague J (2005) Genes that mediate breast cancer metastasis to lung. Nature 436:518-524

Misra S, Heldin P, Hascall VC, Karamanos NK, Skandalis SS, Markwald RR, Ghatak S (2011) Hyaluronan-CD44 interactions as potential targets for cancer therapy. FEBS J 278:1429-1443

Morrison H, Sherman LS, Legg J, Banine F, Isacke C, Haipek CA, Gutmann DH, Ponta H, Herrlich P (2001) The NF2 tumor suppressor gene product, merlin, mediates contact inhibition of growth through interactions with CD44. Genes Dev 15:968-980

Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verastegui E, Zlotnik A (2001) Involvement of chemokine receptors in breast cancer metastasis. Nature 410:50-56

Mundy GR (2002) Metastasis to bone: causes, consequences and therapeutic opportunities. Nat Rev Cancer 2:584-593

Naor D, Nedvetzki S, Golan I, Melnik L, Faitelson Y (2002) CD44 in cancer. Crit Rev Clin Lab Sci 39:527-579

Naor D, Sionov RV, Ish-Shalom D (1997) CD44: structure, function, and association with the malignant process. Adv Cancer Res 71:241-319

Neame SJ, Uff CR, Sheikh H, Wheatley SC, Isacke CM (1995) CD44 exhibits a cell type dependent interaction with triton X-100 insoluble, lipid rich, plasma membrane domains. J Cell Sci 108 (Pt 9):3127-3135

Nguyen DX, Bos PD, Massague J (2009) Metastasis: from dissemination to organ-specific colonization. Nat Rev Cancer 9:274-284

Nishida Y, Knudson W, Knudson CB, Ishiguro N (2005) Antisense inhibition of hyaluronan synthase-2 in human osteosarcoma cells inhibits hyaluronan retention and tumorigenicity. Exp Cell Res 307:194-203

Novak U, Stylli SS, Kaye AH, Lepperdinger G (1999) Hyaluronidase-2 overexpression accelerates intracerebral but not subcutaneous tumor formation of murine astrocytoma cells. Cancer Res 59:6246-6250

Okamoto I, Kawano Y, Matsumoto M, Suga M, Kaibuchi K, Ando M, Saya H (1999a) Regulated CD44 Cleavage under the Control of Protein Kinase C, Calcium Influx, and the Rho Family of Small G Proteins. vol 274, pp 25525-25534

Okamoto I, Kawano Y, Tsuiki H, Sasaki J, Nakao M, Matsumoto M, Suga M, Ando M, Nakajima M, Saya H (1999b) CD44 cleavage induced by a membrane-associated metalloprotease plays a critical role in tumor cell migration. Oncogene 18:1435-1446

Orian-Rousseau V (2010) CD44, a therapeutic target for metastasising tumours. Eur J Cancer 46:1271-1277

Orian-Rousseau V, Chen L, Sleeman JP, Herrlich P, Ponta H (2002) CD44 is required for two consecutive steps in HGF/c-Met signaling. Genes Dev 16:3074-3086

Orian-Rousseau V, Ponta H (2008) Adhesion proteins meet receptors: a common theme? Adv Cancer Res 101:63-92

Padua D, Zhang XH, Wang Q, Nadal C, Gerald WL, Gomis RR, Massague J (2008) TGFbeta primes breast tumors for lung metastasis seeding through angiopoietin-like 4. Cell 133:66-77

Paget S (1989) The distribution of secondary growths in cancer of the breast. 1889. Cancer Metastasis Rev 8:98-101

Patel S, Turner PR, Stubberfield C, Barry E, Rohlff CR, Stamps A, McKenzie E, Young K, Tyson K, Terrett J, Box G, Eccles S, Page MJ (2002) Hyaluronidase gene profiling and role of hyal-1 overexpression in an orthotopic model of prostate cancer. Int J Cancer 97:416-424

Penno MB, August JT, Baylin SB, Mabry M, Linnoila RI, Lee VS, Croteau D, Yang XL, Rosada C (1994) Expression of CD44 in Human Lung Tumors. Cancer Research 54:1381-1387

Perissinotto E, Cavalloni G, Leone F, Fonsato V, Mitola S, Grignani G, Surrenti N, Sangiolo D, Bussolino F, Piacibello W, Aglietta M (2005) Involvement of chemokine receptor 4/stromal cell-derived factor 1 system during osteosarcoma tumor progression. Clin Cancer Res 11:490-497

Perschl A, Lesley J, English N, Hyman R, Trowbridge IS (1995) Transmembrane domain of CD44 is required for its detergent insolubility in fibroblasts. J Cell Sci 108 (Pt 3):1033-1041

Picci P (2007) Osteosarcoma (osteogenic sarcoma). Orphanet J Rare Dis 2:6

Platt VM, Szoka FC, Jr. (2008) Anticancer therapeutics: targeting macromolecules and nanocarriers to hyaluronan or CD44, a hyaluronan receptor. Mol Pharm 5:474-486

Ponta H, Sherman L, Herrlich PA (2003) CD44: from adhesion molecules to signalling regulators. Nat Rev Mol Cell Biol 4:33-45

Posey JT, Soloway MS, Ekici S, Sofer M, Civantos F, Duncan RC, Lokeshwar VB (2003) Evaluation of the prognostic potential of hyaluronic acid and hyaluronidase (HYAL1) for prostate cancer. Cancer Res 63:2638-2644

Psaila B, Lyden D (2009) The metastatic niche: adapting the foreign soil. Nat Rev Cancer 9:285-293

Rall CJN, Rustgi AK (1995) CD44 Isoform Expression in Primary and Metastatic Pancreatic Adenocarcinoma. Cancer Research 55:1831-1835

Ramaswamy S, Ross KN, Lander ES, Golub TR (2003) A molecular signature of metastasis in primary solid tumors. Nat Genet 33:49-54

Riethmacher D, Sonnenberg-Riethmacher E, Brinkmann V, Yamaai T, Lewin GR, Birchmeier C (1997) Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. Nature 389:725-730

Ritter J, Bielack SS (2010) Osteosarcoma. Ann Oncol 21 Suppl 7:vii320-325

Rosen G, Caparros B, Huvos AG, Kosloff C, Nirenberg A, Cacavio A, Marcove RC, Lane JM, Mehta B, Urban C (1982) Preoperative chemotherapy for osteogenic sarcoma: selection of postoperative adjuvant chemotherapy based on the response of the primary tumor to preoperative chemotherapy. Cancer 49:1221-1230

Rosenburg SA, Chabner BA, Young RC, Seipp CA, Levine AS, Costa J, Hanson TA, Head GC, Simon RM (1979) Treatment of osteogenic sarcoma. I. Effect of adjuvant high-dose methotrexate after amputation. Cancer Treat Rep 63:739-751

Rossi R, Gödde A, Kleinebrand A, Riepenhausen M, Boos J, Ritter J, Jürgens H (1994) Unilateral nephrectomy and cisplatin as risk factors of ifosfamide-induced nephrotoxicity: analysis of 120 patients. Journal of Clinical Oncology 12:159-165

Screaton GR, Bell MV, Jackson DG, Cornelis FB, Gerth U, Bell JI (1992) Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. Proc Natl Acad Sci U S A 89:12160-12164

Shaw RJ, McClatchey AI, Jacks T (1998) Regulation of the neurofibromatosis type 2 tumor suppressor protein, merlin, by adhesion and growth arrest stimuli. J Biol Chem 273:7757-7764

Sherman L, Wainwright D, Ponta H, Herrlich P (1998) A splice variant of CD44 expressed in the apical ectodermal ridge presents fibroblast growth factors to limb mesenchyme and is required for limb outgrowth. Genes Dev 12:1058-1071

Sherman LS, Rizvi TA, Karyala S, Ratner N (2000) CD44 enhances neuregulin signaling by Schwann cells. J Cell Biol 150:1071-1084

Shiratori H, Koshino T, Uesugi M, Nitto H, Saito T (2001) Acceleration of lung metastasis by up-regulation of CD44 expression in osteosarcoma-derived cell transplanted mice. Cancer Lett 170:177-182

Shtivelman E, Bishop JM (1991) Expression of CD44 is repressed in neuroblastoma cells. vol 11, pp 5446-5453

Shuster S, Frost GI, Csoka AB, Formby B, Stern R (2002) Hyaluronidase reduces human breast cancer xenografts in SCID mice. Int J Cancer 102:192-197

Sleeman JP, Arming S, Moll JF, Hekele A, Rudy W, Sherman LS, Kreil G, Ponta H, Herrlich P (1996) Hyaluronate-independent metastatic behavior of CD44 variant-expressing pancreatic carcinoma cells. Cancer Res 56:3134-3141

Stamenkovic I, Yu Q (2009) Shedding light on proteolytic cleavage of CD44: the responsible sheddase and functional significance of shedding. J Invest Dermatol 129:1321-1324

Stein U, Walther W, Arlt F, Schwabe H, Smith J, Fichtner I, Birchmeier W, Schlag PM (2009) MACC1, a newly identified key regulator of HGF-MET signaling, predicts colon cancer metastasis. Nat Med 15:59-67

Stiller CA, Craft AW, Corazziari I (2001) Survival of children with bone sarcoma in Europe since 1978: results from the EUROCARE study. European Journal of Cancer 37:760-766

Sugahara KN, Hirata T, Hayasaka H, Stern R, Murai T, Miyasaka M (2006) Tumor cells enhance their own CD44 cleavage and motility by generating hyaluronan fragments. J Biol Chem 281:5861-5868

Ta H, Dass C, Choong P, Dunstan D (2009) Osteosarcoma treatment: state of the art. Cancer and Metastasis Reviews 28:247-263

Termeer C, Benedix F, Sleeman J, Fieber C, Voith U, Ahrens T, Miyake K, Freudenberg M, Galanos C, Simon JC (2002) Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. J Exp Med 195:99-111

Thomas L, Byers HR, Vink J, Stamenkovic I (1992) CD44H regulates tumor cell migration on hyaluronate-coated substrate. J Cell Biol 118:971-977

Tofuku K, Yokouchi M, Murayama T, Minami S, Komiya S (2006) HAS3-related hyaluronan enhances biological activities necessary for metastasis of osteosarcoma cells. Int J Oncol 29:175-183

Toole BP (2002) Hyaluronan promotes the malignant phenotype. Glycobiology 12:37R-42R

Toole BP (2004) Hyaluronan: from extracellular glue to pericellular cue. Nat Rev Cancer 4:528-539

Toole BP (2009) Hyaluronan-CD44 Interactions in Cancer: Paradoxes and Possibilities. Clin Cancer Res 15:7462-7468

Toyama-Sorimachi N, Sorimachi H, Tobita Y, Kitamura F, Yagita H, Suzuki K, Miyasaka M (1995) A novel ligand for CD44 is serglycin, a hematopoietic cell lineage-specific proteoglycan. Possible involvement in lymphoid cell adherence and activation. J Biol Chem 270:7437-7444

Tremmel M, Matzke A, Albrecht I, Laib AM, Olaku V, Ballmer-Hofer K, Christofori G, Heroult M, Augustin HG, Ponta H, Orian-Rousseau V (2009) A CD44v6 peptide reveals a role of CD44 in VEGFR-2 signaling and angiogenesis. Blood 114:5236-5244

Tsukita S, Oishi K, Sato N, Sagara J, Kawai A (1994) ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. J Cell Biol 126:391-401

Turley EA, Noble PW, Bourguignon LY (2002) Signaling properties of hyaluronan receptors. J Biol Chem 277:4589-4592

Valastyan S, Weinberg RA (2011) Tumor metastasis: molecular insights and evolving paradigms. Cell 147:275-292

van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AAM, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R, Friend SH (2002) Gene expression profiling predicts clinical outcome of breast cancer. Nature 415:530-536

Wang LL (2005) Biology of osteogenic sarcoma. Cancer J 11:294-305

Weber GF, Bronson RT, Ilagan J, Cantor H, Schmits R, Mak TW (2002) Absence of the CD44 gene prevents sarcoma metastasis. Cancer Res 62:2281-2286

West DC, Hampson IN, Arnold F, Kumar S (1985) Angiogenesis induced by degradation products of hyaluronic acid. Science 228:1324-1326

Wittig JC, Bickels J, Priebat D, Jelinek J, Kellar-Graney K, Shmookler B, Malawer MM (2002) Osteosarcoma: a multidisciplinary approach to diagnosis and treatment. Am Fam Physician 65:1123-1132

Xu-Dong S, Zan S, Shui-er Z, Li-na T, Wen-xi Y, Feng L, Yang Y (2009) Expression of Ezrin correlates with lung metastasis in Chinese patients with osteosarcoma. Clin Invest Med 32:E180-188

Yamazaki K, Fukuda K, Matsukawa M, Hara F, Yoshida K, Akagi M, Munakata H, Hamanishi C (2003) Reactive oxygen species depolymerize hyaluronan: involvement of the hydroxyl radical. Pathophysiology 9:215-220

Yang J, Weinberg RA (2008) Epithelial-Mesenchymal Transition: At the Crossroads of Development and Tumor Metastasis. Developmental Cell 14:818-829

Yin JJ, Selander K, Chirgwin JM, Dallas M, Grubbs BG, Wieser R, Massagué J, Mundy GR, Guise TA (1999) TGF-β signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. The Journal of Clinical Investigation 103:197-206

Yu Q, Stamenkovic I (1999) Localization of matrix metalloproteinase 9 to the cell surface provides a mechanism for CD44-mediated tumor invasion. Genes Dev 13:35-48

Yu Q, Stamenkovic I (2000) Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes Dev 14:163-176

Yu Q, Toole BP, Stamenkovic I (1997) Induction of apoptosis of metastatic mammary carcinoma cells in vivo by disruption of tumor cell surface CD44 function. J Exp Med 186:1985-1996

Yu WH, Woessner JF, Jr., McNeish JD, Stamenkovic I (2002) CD44 anchors the assembly of matrilysin/MMP-7 with heparin-binding epidermal growth factor precursor and ErbB4 and regulates female reproductive organ remodeling. Genes Dev 16:307-323

Zlotnik A (2004) Chemokines in neoplastic progression. Seminars in cancer biology 14:181-185

Zoller M (2011) CD44: can a cancer-initiating cell profit from an abundantly expressed molecule? Nat Rev Cancer 11:254-267

7 Curriculum Vitae

Name	GVOZDENOVIC Ana
Date of Birth	10.05.1982
Nationality	Serbian
E-mail	gvozdenovic_ana@yahoo.com

Education

Feb 2008 – Apr 2012	PhD Study at the University of Zurich, in Laboratory for Orthopedic Research, Balgrist University Hospital, Zurich, Switzerland within the "Cancer Biology" PhD Program
Oct 2000 – Feb 2007	MSc in Molecular Biology and Physiology, Department of Experimental Biomedicine, Faculty of Biology, University of Belgrade, Serbia Thesis Title: "Amplification of *c-myc* oncogene in ovarian carcinomas"
Sep 1996 – May 2000	The Fifth Belgrade High School, Belgrade Serbia

Publications

Gvozdenovic A, Arlt MJE, Campanile C, Brennecke P, Knut Husmann, Li Y, Born W, Muff R, Fuchs B. CD44 Enhances Tumor Formation and Lung Metastasis in Experimental Osteosarcoma and is an Additional Predictor for Poor Patient's Outcome – manuscript ready for submission

Gvozdenovic A, Arlt MJE, Campanile C, Brennecke P, Knut Husmann, Born W, Muff R, Fuchs B. CD44 Acts as Metastasis Suppressor in an Orthotopic Xenograft Osteosarcoma Mouse Model – manuscript ready for submission

Pastor T, Popović B, **Gvozdenović A**, Boro A, Petrović B, Novaković I, Puzović D, Luković L, Milasin J. Alterations of c-Myc and c-erbB-2 genes in ovarian tumours. Srp Arh Celok Lek. 2009 Jan-Feb;137(1-2):47-51.